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THE UNIVERSITY OF ALBERTA

Protein-Chemical Studies of Band 3, the Anion Transport Protein of Human Erythrocytes

by

Pamela Kathryn Werner

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OF Doctor of Philosophy

Department of Biochemistry

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, a thesis entitled PROTEIN-CHEMICAL STUDIES OF BAND 3, THE ANION TRANSPORT PROTEIN OF HUMAN ERYTHROCYTES submitted by PAMELA KATHRYN WERNER in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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Dated 9 September, 1988

ABSTRACT

The Band 3 protein ($M_r = 95,000$) is an integral membrane protein that facilitates the one-for-one exchange of anions, including chloride and bicarbonate, across the human erythrocyte membrane. My thesis research has included the characterization of the structure of purified Band 3, both in detergent solutions and in lipid vesicles, and investigations, using chemical modification, of the role of carboxyl residues in anion exchange and of the reactivity of sulfhydryl groups in Band 3.

The aim of the first project was the characterization of the Band 3 protein's structure after its isolation, and after its reinsertion into phospholipid vesicles. Band 3 was purified in the nonionic detergent octyl glucoside (OG), and its structure was assessed using various hydrodynamic and spectroscopic techniques. OG induced small changes in the secondary structure of Band 3, as determined by circular dichroism, as well as a dramatic increase in the aggregation state. The affinity of binding of the anion transport inhibitor 4-benzamido-4'-aminostilbene-2,2'-disulfonate (BADS, K_i=1 μM) was irreversibly decreased by an order of magnitude, indicating a structural change had occurred. The results show that OG alone is not a suitable detergent for reconstitution of fully functional Band 3. Band 3 reconstituted into lipid vesicles using a mixture of OG and octaethyleneglycol mono-n-dodecyl ether ($C_{12}E_8$) had normal BADS binding affinity and circular dichroism measurements indicated a high content of α-helix.

Treatment of erythrocytes with the carboxyl-selective reagent, 1-ethyl-3-[3-(trimethylammonio)propyl] carbodiimide (ETC) can produce inhibition of anion transport by two distinct mechanisms. One mechanism was shown to be due not to protein carboxyl modification, but to carbodiimide-activated citrate modification of an essential lysine residue in Band 3. This modification has potential use in the identification of a lysine residue that may be involved in the transport mechanism, and the characteristics of the transport by the modified transporter may clarify the role of this lysine in anion exchange.

The characteristics of the other mode of inhibition are consistent with its involving modification of a carboxyl group. Addition of hydrophobic nucleophiles during treatment with ETC in chloride media resulted in stabilization of the otherwise reversible inhibition, indicating that the carbodiimide- and the nucleophile-adduct both result in inhibition. Stilbenedisulfonates do not afford protection against the inhibition. These results provide strong evidence that a carboxyl group outside the inhibitor binding region is important in anion exchange.

A study investigating the reactivity and accessibility of sulfhydryl groups indicated that an N-ethylmaleimide (NEM)-unreactive cysteine residue in Band 3 is accessible to a matrix-bound sulfhydryl reagent, suggesting that it is not buried within the solubilized protein. Further groups in Band 3 are reactive with NEM after solubilization in $C_{12}E_8$.

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LIST OF ABBREVIATIONS AND SYMBOLS

$A(\lambda)$	absorption as a function of wavelength
A_f	apparent absorption due to absorption flattening
A_s	apparent absorption due to scattering
A_{sln}	absorption of a solubilized vesicle suspension
A_{susp}	absorption by a suspension of vesicles
ATP	adenosine 5'-triphosphate
BADS	4-benzamido-4'-aminostilbene-2,2'-disulfonate
BSSS	bis(sulfosuccinimidyl suberate)
$C_{1/2}$	concentration producing a half-maximal fluorescence change
$C_{12}E_8$	octaethyleneglycol mono-n-dodecyl ether
$C_{12}E_9$	nonaethyleneglycol mono-n-dodecyl ether
CD	circular dichroism
ChC-35	the C-terminal 35,000 dalton chymotryptic fragment of Band 3
cmc	critical micellar concentration
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonate
DNDS	4,4'-dinitrostilbene-2,2'-disulfonate
DTNB	5,5'-dithiobis (2-nitrobenzoate)
EAC	1-ethyl-3-(4-azonia-4,4'-dimethylpentyl) carbodiimide
EDTA	ethylene diamine tetraacetate
ETC	1-ethyl-3-[3(trimethylammonio)propyl] carbodiimide
F	fluorescence
F_{max}	maximal fluorescence
H_2DIDS	1,2- (2,2'-disulfo-4,4'-diisothiocyano) diphenylethane
$^3H_2$DIDS	[^{3}H]dihydroDIDS; 1,2-ditritio(2,2'-disulfo-4,4'-diisothiocyano) diphenylethane

I_{50}	concentration of inhibitor that produces 50% inhibition
K_{av}	partition coefficient
kcal	kilocalorie
K_d	dissociation constant
kDa	kilodalton
K_i	inhibitor binding constant
MES	2-(*N*-morpholino)ethanesulfonic acid
M_r	relative molecular mass
Mc	molecular weight of a protein-detergent complex
NCh-60	the N-terminal 60,000 dalton chymotryptic fragment of Band 3
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
NTCB	2-nitro-5-thiocyanobenzoic acid
NTyr	nitrotyrosine
-OEt	-ethyl ester
OG	n-octyl-β-D-glucopyranoside (octyl glucoside)
PBS	phosphate buffered saline, 150 mM NaCl, 5 mM sodium phosphate, pH 7.0 to 8.0, as indicated
pCMB	para-chloromercuribenzoic acid
pCMB-Sepharose	[[p-(chloromercuri) benzamido]ethylene]amino-Sepharose 4B.
pCMBS	para-chloromercuribenzene sulfonate
PheOEt	phenylalanine ethyl ester
P_i	inorganic phosphate
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PL	phospholipid
PLP	pyridoxal phosphate
R_f	retention factor; the ratio of the distances traveled by a molecule and by the dye front

R_s	Stokes radius
S	Svedberg (units of sedimentation), 1 S=10^{-13} sec
$s_{20,w}$	standard sedimentation coefficient (standardized to the solvent water at 20 °C)
$s^{\circ}_{20,w}$	intrinsic sedimentation coefficient
$s^{\circ}_{20,w,c}$	intrinsic sedimentation coefficient of the complex
SDS	sodium dodecyl sulfate
SITS	4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
δ_D	g detergent bound per g protein
λ	wavelength
$[\theta]$	molar ellipticity
$\bar{\upsilon}$	partial specific volume
$\bar{\upsilon}_d$	partial specific volume of the detergent
$\bar{\upsilon}_p$	partial specific volume of the protein
$\bar{\upsilon}_c$	partial specific volume of the protein-detergent complex

I. INTRODUCTION

A. Survey of Membrane Transport and Membrane Protein Structural Principles

Biological membranes act as a boundary between the cell and its environment, and between membrane-bound compartments within a cell. The boundary function is provided by lipids, which associate to form a bilayer that acts as a permeability barrier such that polar solutes have very limited permeability. A selective permeability of the membrane is provided by the other main component of biological membranes, the membrane proteins. Membrane proteins can have functions in addition to controlling the transport of solutes across the membrane, such as acting as receptors for transmitting intercellular signals, as anchors for cell-surface carbohydrates that act in recognition between cells, and as anchors for enzymes.

In the fluid-mosaic model of membranes (Singer and Nicolson, 1972) the lipid components, including phospholipids, cholesterol and glycolipids, associate in a bilayer with their hydrophobic parts associating in the inner part of the membrane, and their polar head groups facing the aqueous environment. Integral membrane proteins are embedded in the bilayer, partly associating with the nonpolar region through hydrophobic interactions, and partly exposed to the aqueous environment at one or both sides of the membrane. These proteins require the use of detergents or organic solvents to remove them from their association with the bilayer. Peripheral membrane proteins are often found associated with the membrane by interaction with integral proteins, or with lipid headgroups. This class of proteins can usually be removed from the membrane by relatively mild treatment such as a change in pH or ionic strength. Another class of membrane proteins has recently been described (Low and Saltiel, 1988) , and these proteins are associated via a covalent linkage to phosphatidyl inositol, but are otherwise more like soluble proteins rather than integral membrane proteins. The membrane association of phosphatidylinositol-linked proteins can be disrupted by treatment with phospholipase C. Examples of this class are: acetylcholinesterase and 5'-nucleotidase.

The integral membrane proteins can be categorized (Guidotti, 1986) as those having only a single traverse of the polypeptide across the bilayer, and those having multiple spans. The first type are often represented by membrane-bound enzymes and receptors. The second type are often involved in transport of solutes across the bilayer (referred to as permeases, or transporters). For some transporters, transport occurs only in response to existing concentration gradients (facilitated diffusion), while for others it requires the simultaneous input of energy (active transport), in the form of light energy, hydrolysis of ATP, or the dissipation of an existing electrochemical gradient. Examples of well-characterized transporters include: Na^+/K^+-ATPase, Ca^{2+}-ATPase, anion transporter of erythrocytes, rhodopsin, glucose transporter, Na^+ channel, nicotinic acetylcholine receptor, bacteriorhodopsin, and the lactose permease. The concept of a protein catalyzing the movement of solute across the permeability barrier is analogous to the action of an enzyme, and is thought to be brought about by small conformational changes in the protein. Integral membrane proteins are fixed in their orientation in the bilayer, and can not rotate. Thus, some of the functionally important parts of transport proteins are likely to be in the transmembrane regions.

The considerations determining the folding and stabilization of integral membrane proteins are very different than for soluble proteins. The hydrophobicity of the bilayer core necessitates a structure for the hydrophobic domain in which the peptide structure is maximally hydrogen bonded (Tanford, 1973; Guidotti, 1986). This can be readily accomodated by an α-helical conformation. The classic example of structure of an integral membrane protein is that of bacteriorhodopsin from *Halobacterium halobium*. A three-dimensional model of bacteriorhodopsin in two-dimensional ordered arrays was determined using image reconstruction of electron micrographs (Unwin and Henderson, 1975). It consists of a bundle of 7 helices oriented roughly perpendicularly to the plane of the bilayer. The multi-helical motif is found in another membrane protein for which a crystal structure has been determined. The integral membrane L and M subunits of the photosynthetic reaction center of *Rhodopseudomonas viridis* have, together, 10 aligned α-helices, each of 24 to 30 residues in length. Although certain intrinsic membrane proteins have been shown to have β-structure (e.g., the bacterial porins, Paul and Rosenbusch, 1985), it is thought that a series of helices roughly perpendicular to the bilayer is a general structural feature common to the multiple-spanning intrinsic membrane proteins. Connections between multiple helices may consist of a β-turn, which is likely to occur at the aqueous surface of the protein, outside the hydrophobic core of the membrane.

The existing techniques to study integral membrane protein structure have limitations. At present, very few crystal structures have been solved, and crystallization must be carried out in the presence of amphiphiles. Recently, however, much progress in crystallization of membrane proteins has been made and X-ray crystallography of membrane-bound proteins may be just coming of age. NMR , infrared, circular dichroism, and absorption spectroscopy have all been applied to membrane proteins, but sometimes require special precautions when studying these hydrophobic proteins. For example, such techniques are often applicable only to detergent-solubilized proteins rather than the membrane-bound form, because the size of the absorbing particle must be small. For many membrane proteins, the first difficulty is to obtain sufficient amounts of purified protein in native form in order to do structural studies.

Because of their hydrophobic nature, integral membrane proteins require detergents to solubilize them from the membrane and to keep them soluble during their isolation. Detergents, like phospholipids, are amphiphilic compounds, having a hydrophobic and a hydrophilic part. Because of this property, above a certain concentration (the critical micellar concentration) of detergent in an aqueous solution the detergent molecules tend to associate to form larger structures called micelles. Detergents both disrupt the structure of phospholipid bilayers as well as substitute for the lipid in associating with the hydrophobic parts of the integral membrane proteins.

Generally, the same types of chromatographic techniques used in the purification of soluble proteins are applicable to membrane proteins if done in the presence of nonionic detergents. The large hydrophobic surface of integral membrane proteins can be demonstrated by the fact that after solubilization with nondenaturing detergents, they bind substantial amounts of detergent (Clarke, 1975). Whether as a preliminary for reconstitution, or for further studies of the detergent-solubilized protein, it is important to ensure that the functional and structural integrity of the protein is retained in a particular detergent. The approaches to studying membrane proteins solubilized in detergents have been reviewed (Tanford and Reynolds, 1976; Møller *et al.*, 1986). These include probes of functional integrity, such as measurements of an associated enzymatic activity, or measuring the binding of specific ligands. Information about the protein's secondary structure can be determined using circular dichroism, and specific structural features such as intrinsic chromophore absorption may be available for study. In addition, the overall molecular size, including oligomeric structure, can be assessed using crosslinking, gel filtration or analytical ultracentrifugation in the presence of detergents.

In order to measure the transport function of a purified transport protein it is necessary to re-insert it into a sealed phospholipid bilayer (vesicles or monolayers), which usually involves replacing the protein-associated detergents with phospholipids. A number of extensive reviews are available covering the principles of and examples of attempts at reconstitution of transport protein function (Cabantchik and Darmon, 1985; Eytan, 1982; Etemadi, 1985). Reconstitution is laborious and not always reproducible, so it is useful to find additional methods to assess the functional or conformational integrity of the protein in detergent solution. A major advantage of having a reconstituted preparation is that the functional properties of an individual protein component can be studied in a controlled environment. In addition, the lipid composition of the vesicles can be systematically varied to study the effects of the lipid components of the membrane on transport.

The anion transport protein of the human erythrocyte membrane is one of the best characterized eukaryotic transport proteins. There are many less well characterized examples of cellular anion transport, some of which may be structurally and functionally related to the erythrocyte anion transporter (for examples, see Wieth and Brahm, 1985; Knauf, 1986). Detailed study of the mechanism and structural aspects of the erythrocyte anion exchange protein may be useful in the characterization of other anion transport processes and is likely to extend the existing general principles of protein-mediated membrane transport.

B. Anion Exchange in Red Cells

1. Physiological Importance of Anion Exchange

The exchange of chloride and bicarbonate across the erythrocyte membrane is important in increasing the carbon dioxide carrying capacity of the blood. The overall process of transport and excretion of CO_2 involves interconversion of CO_2 and HCO_3^-, which is catalyzed inside the erythrocyte by carbonic anhydrase. CO_2 readily diffuses across the membrane, but the charged HCO_3^- must cross by a protein-mediated process. In the systemic capillaries, the transporter-mediated exchange of intracellular HCO_3^- for extracellular Cl^- results in removal of HCO_3^- from the cell, which allows continued conversion of CO_2 to HCO_3^- by carbonic anhydrase. The transport of bicarbonate into the plasma also increases the volume available for carrying the bicarbonate. The reverse processes occur in the pulmonary capillaries, during release of the gaseous CO_2, with the exchange transporter allowing HCO_3^- to enter the cell to be converted to CO_2. The contribution of the anion exchange process to this function has been evaluated and summarized (Wieth *et al.*, 1982a; Lowe and Lambert, 1983). By making the plasma volume available to dissolved bicarbonate, the CO_2 carrying capacity of the blood is increased about 2-fold. Anion exchange is also thought to be involved in regulating cellular pH, with HCO_3^- exit allowing cells to recover from an alkaline load (Olsnes *et al.*, 1986).

2. Properties of Anion Exchange

As an example of a facilitated diffusion process, the anion exchange transport system responds to existing concentration gradients, increasing in transport rate with increasing concentration of anion, but showing saturation of the rate at high substrate concentrations. The process also shows a fairly broad, but definite substrate specificity. Although the physiologically relevant anions are Cl^- and HCO_3^-, other halides (Br^-, I^-) and oxyanions (phosphate, sulfate), and even larger organic anions are transported by the same transport pathway (Knauf, 1979); they all show mutual competition for transport, and their transport is inhibited by the same reagents. The rates of transport of different anions do not follow the series of anion binding affinities, and vary over a range of 10^4, with Cl^- and HCO_3^- having the highest transport rates (Knauf, 1979). Both the saturation and substrate selectivity indicate anion binding sites must be involved in the transport process.

Anion transport in erythrocytes is electrically silent. Although the measured exchange flux is high, the anion conductance of the membrane is about four orders of magnitude lower (Knauf *et al.*, 1977). This indicates that the anion transport must be a strict exchange-only process involving one anion being transported in one direction in exchange for one in the opposite direction. Even the low levels of conductive flux, though, seem to be catalyzed by the same protein (Knauf *et al.*, 1977). Further discussion of how these facts can be incorporated into a catalytic mechanism will be given later.

Rates of anion exchange are very rapid, and can be as high as 10^5 ions·sec^{-1} per transporter molecule, for Cl^- at 37 °C (Brahm, 1977). This is almost as high as the turnover number of carbonic anhydrase, an enzyme with a very high turnover number of 6 x 10^5 substrate molecules·sec^{-1} per enzyme molecule (Lehninger, 1975). The rates vary, of course, depending on the particular ions being transported. The transport rate is also highly temperature dependent. Activation energies measured for Cl^- and Br^- exchange are about 25 to 30 kcal·mol $^{-1}$, but somewhat less at temperatures above about 15 °C (Brahm, 1977). This activation energy is not due to anion binding, but reflects some energy barrier to the translocation step (Brahm, 1977).

Detailed studies of the kinetics of anion exchange indicate, by analogy to two-substrate enzymes, that the one-for-one exchange occurs by a ping-pong mechanism, as opposed to a simultaneous mechanism, in which the steps of binding, translocation and dissociation of an anion being transported in one direction occur before the steps of an anion moving in the opposite direction (Knauf, 1979; Gunn and Fröhlich, 1979). In accordance with this, it is possible to produce distinguishable inward-facing and outward-facing conformations of the transporter (Knauf *et al.*, 1980; Passow *et al.*, 1980b; Furuya *et al.*, 1984), and to choose conditions to recruit the transport sites to one side of the membrane or the other (Jennings, 1980; Falke *et al.*, 1984b). Discussion of the kinetic model and recruitment phenomena will be covered in more detail in the "Mechanistic Aspects" section.

3. Identification of Band 3 as the Anion Exchange Protein

a. Erythrocyte Membrane Proteins. The erythrocyte membrane represents one of the best characterized eukaryotic cell membranes -- it is also one of the simplest in composition and the most amenable to study. The plasma membrane, which is the only membrane present in mammalian erythrocytes, is readily isolated by hypotonic lysis of the cells, and separation of the membranes from the cytoplasmic contents by centrifugation (Steck, 1974). The resulting ghost membranes are composed of about half protein, by weight (Steck, 1974), but the protein content is relatively simple. Separation of the erythrocyte membrane proteins by their relative molecular weights, using SDS polycrylamide gel electrophoresis, indicates about 10 predominant protein species. They are, by convention (Fairbanks *et al.*, 1971), designated by number of the staining band in order of decreasing molecular weight, as shown in Figure I.1.

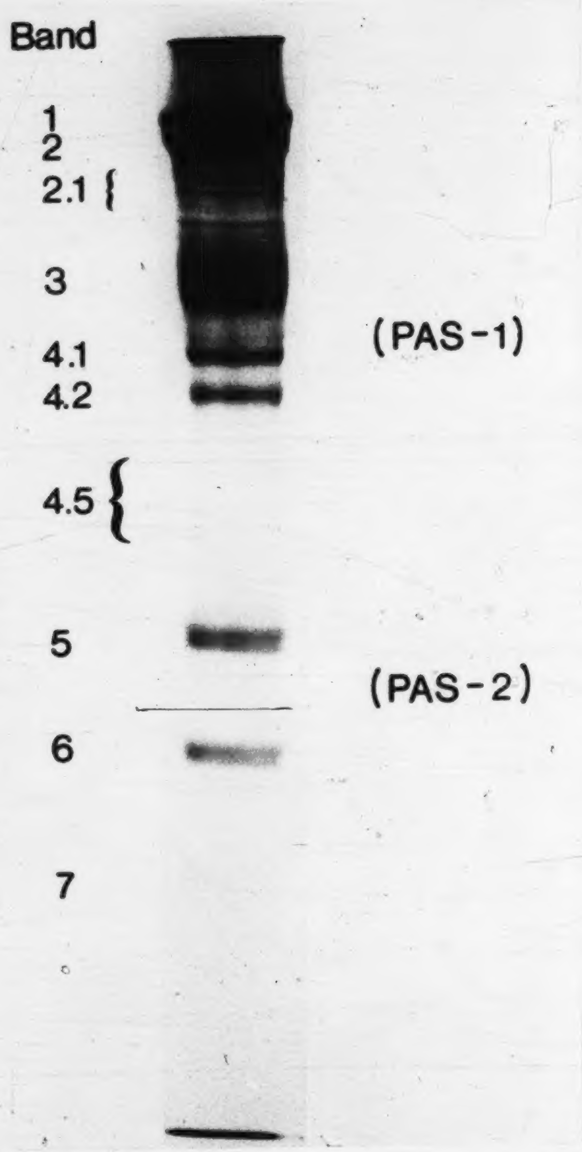

Figure I.1 Separation of human erythrocyte membrane proteins by SDS polyacrylamide gel electrophoresis. Electrophoresis was performed in a gel containing 10% acrylamide using the Laemmli buffer system. Numbers on the left indicate the the major bands appearing after staining with Coomassie Blue. The relative location of the major bands that would appear upon staining with periodic acid-Schiff reagent is indicated on the right.

Several of the proteins of the red cell membrane are peripheral membrane proteins. That is, they are not associated with the hydrophobic core of the bilayer, but are associated with the membrane *via* interactions with integral components. Most of these proteins comprise the cytoskeletal network that lines the cytoplasmic side of the membrane and controls the cell shape and deformability (Branton *et al.*, 1981), and include: Bands 1 and 2, the α- and β-subunits of spectrin;, ankyrin, (band 2.1); Bands 4.1 and 4.2; and Band 5, which is actin. Band 6, glyceraldehyde 3-phosphate dehydrogenase, is a peripheral membrane protein, but is not part of the cytoskeleton.

The integral membrane proteins are proteins that can be removed from the membrane only by treatment with detergents or organic solvents, and are thus thought to interact directly with the hydrophobic core. The major integral protein is Band 3[1], which accounts for about 25% by weight of the membrane proteins. The Band 3 region consists chiefly of the anion exchange protein, but also contains very minor amounts of other proteins unrelated to anion transport, such as the Na^+/K^+ ATPase (Avruch and Fairbanks, 1972), Ca^{2+}-ATPase and acetylcholinesterase (Bellhorn *et al.*, 1970). The Band 4.5 region contains at least two integral membrane proteins, a glucose transporter (Lienhard *et al.*, 1983; Mueckler *et al.*, 1985) and a nucleoside transporter (Wu *et al.*, 1983). Band 7 is an integral protein of unknown function. In addition, there are several integral sialoglycoproteins that are not visualized by staining with Coomassie Blue stain, but which have been characterized by their staining with periodic acid-Schiff reagent (PAS) and Stains-All (Steck, 1974; Furthmayr, 1978). The major sialoglycoprotein is glycophorin A (M_r=31,000) (Bretscher, 1971), which migrates in most gel systems at positions between Bands 5 and 6 (PAS-2) for the monomer, and below Band 3 (PAS-1) for the dimer. The number of copies of glycophorin A is estimated to be about half the number of Band 3.

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[1] An alternative nomenclature proposed for this protein is capnophorin, which has its origin in the Greek for "smoke" carrier (Wieth *et al.*, 1982b; Wieth and Bjerrum, 1983). Throughout this document, however, we use the term Band 3 .

Glycophorins B and C have sequences distinct from glycophorin A and are minor components of the membrane (Furthmayr, 1978). They are all examples of cell surface antigen-carrying membrane proteins.

b. Chemical Identification. The identification of the protein migrating at the position of Band 3 as the component responsible for anion transport across the erythrocyte membrane was achieved using amino-reactive compounds that inhibited anion exchange (Knauf and Rothstein, 1971) and selectively labeled Band 3 (Cabantchik and Rothstein, 1974; Ho and Guidotti, 1975). The binding of several of a family of related compounds, the stilbenedisulfonates, was found to be proportional to inhibition of transport (Lepke *et al.*, 1976; Ship *et al.*, 1977). These inhibitors were important in providing a marker for the protein during isolation, since there was no associated enzymatic activity that could be measured in solubilized Band 3 preparations, and inhibitors continue to be useful tools in Band 3 research.

c. Functional Reconstitution. The reconstitution of the essential qualitative features of anion transport by insertion of purified Band 3 protein into lipid vesicles (Wolosin *et al.*, 1977; Wolosin *et al.*, 1980; Cabantchik *et al.*, 1980) has been sufficient to definitively assign the transport function to Band 3 protein alone. Several of the more specific characteristics of the transport activity of these preparations, however, differed from the transport measured in erythrocytes. Some of the earlier reports (Rothstein *et al.*, 1975; Ross and McConnell, 1977, 1978) used protein preparations that were not highly purified, or appeared to be denatured. Recent, continuing efforts in several labs have been directed at developing a reconstitution method that gives highly reproducible anion transport that approximates the activity in the native state. Such a preparation would be required for carrying out structure-function studies on purified Band 3 protein in a native-like environment.

The criteria for determining the adequacy of a particular reconstitution are production of reproducible and native values for : transport rate; temperature dependence;

pH-dependence; susceptibility to appropriate concentrations of specific inhibitors, and other indicators that transport is specific for Band 3; substrate anion selectivity and affinities. A major problem with all the preparations to date seems to be the result of heterogeneity in the vesicle populations, for instance, in the trapped volume per vesicle, and the protein content per vesicle. Although most of the reported temperature dependencies for the reconstituted preparations were in an acceptable range (from 20 - 36 kcal/mol), the reported transport rates were quite variable. Some of the reports (Wolosin *et al.*, 1980; vanHoogevest *et al.*, 1983) even noted that independent preparations of vesicles, prepared in an identical manner, produced variable rates of transport. The transport rate is the parameter that is the most susceptible to influence by heterogeneity in the vesicle population. Contributing to this heterogeneity, many of the preparations were apparently plagued by high nonspecific leak fluxes (Köhne *et al.*, 1983; van Hoogevest *et al.*, 1983; Cabantchik *et al.*, 1983). The cases that reported stilbenedisulfonate inhibitory potency in the vesicles noted that it was lowered, requiring higher concentrations of inhibitor to produce maximal inhibition (Wolosin *et al.*, 1980; Köhne *et al.*, 1981; van Hoogevest *et al.*, 1983).

The reconstitution reported by Schubert and coworkers (Scheuring *et al.*, 1986) has characteristics of anion exchange that more closely resemble those in the erythrocyte than any of the previous reports. They have done a thorough characterization to show that the essential features of transport are retained in this preparation: rates of transport in the reconstituted proteoliposomes are comparable to the rates in cells (≅ 20 sulfate ions/min/Band 3 molecule at 30 °C), but still show substantial variability in this value from one preparation to the next; a normal and reproducible activation energy (33-35 kcal/mol); inhibition by normal concentrations of the inhibitors H_2DIDS and flufenamate; a bell-shaped pH dependence with a maximum at about pH 6.5; much slower net transport than exchange. In addition, the Band 3 was oriented in an almost exclusively right-side-out orientation, as demonstrated by inhibition of 98% of the transport by externally added H_2DIDS. The more recently published improvements on this method (Scheuring *et al.*, 1987, 1988) allow a less heterogenous vesicle population to be produced, eliminating sucrose-leaky vesicles. Additional improvement in reproducibility of preparation, especially in the homogeneity in the sizes and protein contents of the vesicles, are warranted in order to achieve the reproducible rates of transport that are necessary to make comparative measurements. This preparation comes very close to being suitable for performing structure-function studies, assessing the effects of, for example, lipids, or protein modifications, on transport in this defined system.

C. Band 3 Protein Structure

Several of the recently published reviews provide excellent summaries of the sequence and the protein chemical information for human Band 3 (Jennings, 1985; Knauf, 1986), especially those including comparisons to the primary structure of murine Band 3 (Kopito and Lodish, 1985a,b; Jay and Cantley, 1986). In the brief summary provided here the content of these reviews is presented, with the addition of some recent data on the structure of human Band 3.

1. Monomer Size and Oligomeric Structure

The migration of the Band 3 protein in SDS polyacrylamide gel electrophoresis indicates a molecular weight of about 95,000 daltons for the monomer (Fairbanks *et al.*, 1971). The size of the protein in its native form has been determined under two types of conditions -- *in situ*, in the erythrocyte membrane, and after extraction from the membrane in detergent-solubilized form. Results under both conditions indicate a tendency for Band 3 molecules to exist as oligomers (dimer and higher oligomers). The work relating to the oligomeric state of Band 3 has been comprehensively reviewed (Jennings, 1984), and will be only briefly reiterated here.

Evidence from several techniques suggests that Band 3 exists as a mixture of dimer and perhaps also tetramer in the intact erythrocyte membrane: the number of intramembrane particles observed in freeze-fracture electron microscopy are consistent with their representing dimers and tetramers; chemical crosslinking performed in cells generally produces dimers. Additional crosslinking experiments as well as studies of spectroscopic probes attached to Band 3 have been done on erythrocyte ghosts and support the presence of dimers, and sometimes higher oligomers. Most studies support the absence of monomeric Band 3 in the membrane, although monomers may exist in dilute solutions of Band 3 in detergent. Radiation inactivation on cells and ghosts has indicated a target size of 200 kDa for inactivation of anion transport (Cuppoletti *et al.*, 1985), though a smaller size for stilbenedisulfonate binding in ghosts (Verkman *et al.*, 1986).

Band 3 appears to maintain some degree of its oligomeric state even when it is solubilized from the membrane by detergents, as indicated using the techniques of analytical ultracentrifugation, gel filtration, and nondenaturing gel electrophoresis (all in detergents). The current model of the state of association in detergent is of a reversibly associating mixture of monomer, dimer and tetramer (Pappert and Schubert, 1983). Some reports indicate a stable dimeric structure (Yu and Steck, 1975b; Clarke, 1975), or a mixture of stable dimers and tetramers (Nakashima and Makino, 1980b; Nakashima *et al.*, 1981), or even monomer and dimer (Lukacovic *et al.*, 1981). Others ascribe this stability to oxidizing impurities in the detergents (Schubert *et al.*, 1983). Even higher states of association have been reported for Band 3 in aqueous solution after exposure to acetic acid (Dorst and Schubert, 1979) but are of questionable physiological relevance. Comparing these results, there seems to be a small but apparent influence of different nonionic detergents on the oligomeric state. The finding that the membrane domain maintains its dimeric association even after solubilization (Reithmeier, 1979) may suggest that portions within the membrane domain are involved in the oligomerization.

The high protein density in the membrane and the cytoskeletal interactions surely influence the association of Band 3 in the native membranes. The relative concentration of Band 3 in the membrane is approximately 100 mg/mL, yet the dilution occuring upon solubilization apparently does not destroy the tendency for oligomeric interaction.

Together, these results indicate that the probable native oligomeric state of Band 3 in the membrane is of dimers which can further associate to form tetramers. Preliminary investigations of the parts of the protein involved in oligomeric interaction have been included in several of the above-mentioned studies, as well as by others (Staros and Khakkad, 1983; Jennings and Nicknish, 1985), but are not yet definitive. The possible functional role of oligomerization is well summarized by Jennings (1984). Each monomer of Band 3 seems to function independently in anion transport, but there is evidence of interactions between the subunits as observed in the binding of large stilbenedisulfonates.

2. Amino Acid Sequence and Major Proteolytic Fragments

Band 3 has been well characterized by proteolytic and chemical fragmentation (Steck *et al.*, 1978; many other references, which are summarized in Figure I.2, in Jay and Cantley, 1986, and in Knauf, 1986). The major proteolytic fragments of human Band 3 are summarized in the upper section of Figure I.2. In referring to these fragments throughout the text, we have adopted the nomenclature[2] suggested by Jay and Cantley (1986), by which the chemical or proteolytic origin of generation of the fragment, as well as the size, are denoted (see the legend to Figure I.2). Cleavage with trypsin at the cytoplasmic side of the membrane generates a large fragment NTr-40, which is not membrane-bound, and the membrane-bound TrC-55 (Steck *et al.*, 1978). These fragments represent structurally and functionally independent domains of Band 3 protein, the cytoplasmic domain, NTr-40, being involved in both the binding of cytoskeletal proteins and glycolytic enzymes, and the membrane domain, TrC-55, mediating the transport function (Appell and Low, 1982). The fragment NTr-40 can be further cleaved to produce

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[2] The nomenclature is a three part abbreviation in which the first part is an abbreviation for the origin of the N-terminus of the fragment, the second part is an abbreviation for the origin of the C-terminus of the fragment, followed by the approximate size of the fragment in kDa. The legend to Figure I.1 contains details of the abbreviations used for the cleavages.

NTr-23 and TrTr-20, or by S-cyanylation to produce NSc-22 (Steck *et al.*, 1978). Chymotrypsin treatment of erythrocytes produces two fragments, NCh-60 and ChC-35, both of which are membrane bound. This same treatment, followed by trypsin treatment at the cytoplasmic side, forms the sub-fragments TrCh-17 and ChC-35 from TrC-55. This cleavage does not affect anion transport (Grinstein, Ship and Rothstein, 1978). There have been additional studies of fragmentation of TrCh-17 which are not depicted in the figure (Ramjeesingh, Gaarn and Rothstein, 1980, 1981; Ramjeesingh and Rothstein, 1982; Mawby and Findlay, 1982). Additional cleavage sites within ChC-35 are shown in this fragment map, including sites of papain cleavage near the N-terminus of ChC-35, which affect anion transport (Jennings and Adams, 1981), and generate ChPa-8 and PaC-28. A second cytoplasmic site of trypsin cleavage occurring under conditions of low ionic strength, and generating the fragment TrC-20 has been recently described (Jennings, Anderson and Monaghan, 1986). TrC-55, ChC-35 and TrC-20 all extend to the C-terminus, as defined by C-terminal site-specific antibody binding (Lieberman and Reithmeier, 1988). More details of the sites of cleavage are denoted in the model of the folding (Figure I.3).

Prior to 1985 under 40% of the sequence of human Band 3 protein had been determined using protein sequencing methods. About two-thirds of this was from the N-terminal, cytoplasmic domain (Kaul *et al.*, 1983). The portions that are intimately associated with the membrane have been notoriously difficult to purify and sequence using classical techniques due to their hydrophobicity. In 1985 the sequence of a full-length cDNA for murine Band 3 was published by Kopito and Lodish (1985a). The protein sequence deduced from this is 929 amino acid residues long, which would correspond to a molecular mass of 103,000, and is close to the molecular sizes estimated by gel electophoresis for the human and the murine proteins (Kopito and Lodish, 1985a). The schematic in the lower part of Figure I.2 illustrates the approximate locations of the fragments of human Band 3 that have been sequenced, in alignment with the deduced

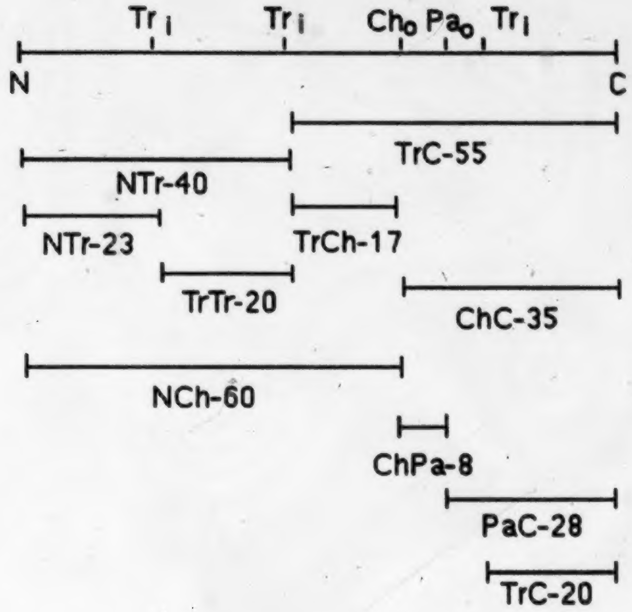
Figure I.2 Band 3 protein sequence and proteolytic fragments

A. Map of the major proteolytic fragments of human Band 3. The sites in the intact Band 3 are denoted by a mark with the abbreviation of the protease, and a subscript indicating the side of the membrane at which the cleavage occurs, either inside (i) or outside (o). The nomenclature of the fragments is that suggested by Jay and Cantley (1986), as a three-part abbreviation, in the format NC-#, where N and C represent the abbreviations for the origin of the N-terminus and the C-terminus of the fragment , respectively, followed by the approximate size of the fragment, in kDa. For example, TrCh-17 describes the fragment generated by a trypsin cleavage at its N-terminus, a chymotrypsin cleavage at its C-terminus, and having an apparent molecular weight of 17 kDa. The abbreviations used are: Ch, chymotrypsin; Tr, trypsin; N, N-terminus; C, C-terminus; Pa, papain. The original references describing these fragments can be found in Jay and Cantley (1986), and details of the sites of the cleavages are represented in Figure I.3. Additional fragments (i.e., CNBr or NTCB-generated) have not been included here.

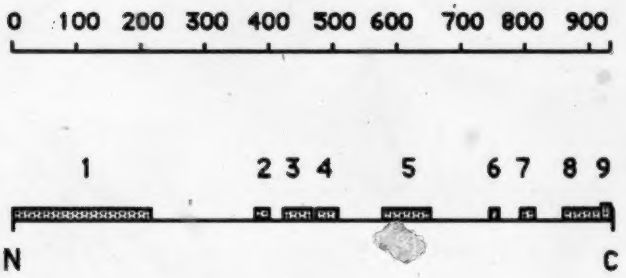
B. Schematic of the approximate locations of the protein sequences known for human Band 3, in alignment with the deduced murine Band 3 sequence. The scale at the top represents the corresponding murine residue numbers, and the numbered, shaded lines represent the fragments of the human protein, as described below. Some sequences that could not be aligned or found within the murine sequence have not been included.

region #	corresponding murine residues	Reference
1	1 - 214	Kaul *et al.*, 1983
2	375 - 380	Mawby and Findlay, 1982
	376	Jenkins and Tanner, 1977
3	417 - 449	Brock and Tanner, 1986
4	456 - 492	Mawby and Findlay, 1982
5	577 - 648	Brock, Tanner and Kempf, 1983
	568 - 572	Jennings and Adams, 1981; Jennings and Nicknish, 1985
	577 - 592	Jennings *et al.*, 1984
6	762 - 771	Jennings *et al.*, 1986
7	794 - 813	Brock and Tanner, 1986
8	852 - 929	Kawano *et al.*, 1988
9	918 - 929	Lieberman *et al.*, 1988

A


Tr i
Tr i
Ch o
Pa o
Tr i
N
C
TrC-55
NTr-40
NTr-23
TrCh-17
TrTr-20
ChC-35
NCh-60
ChPa-8
PaC-28
TrC-20

B

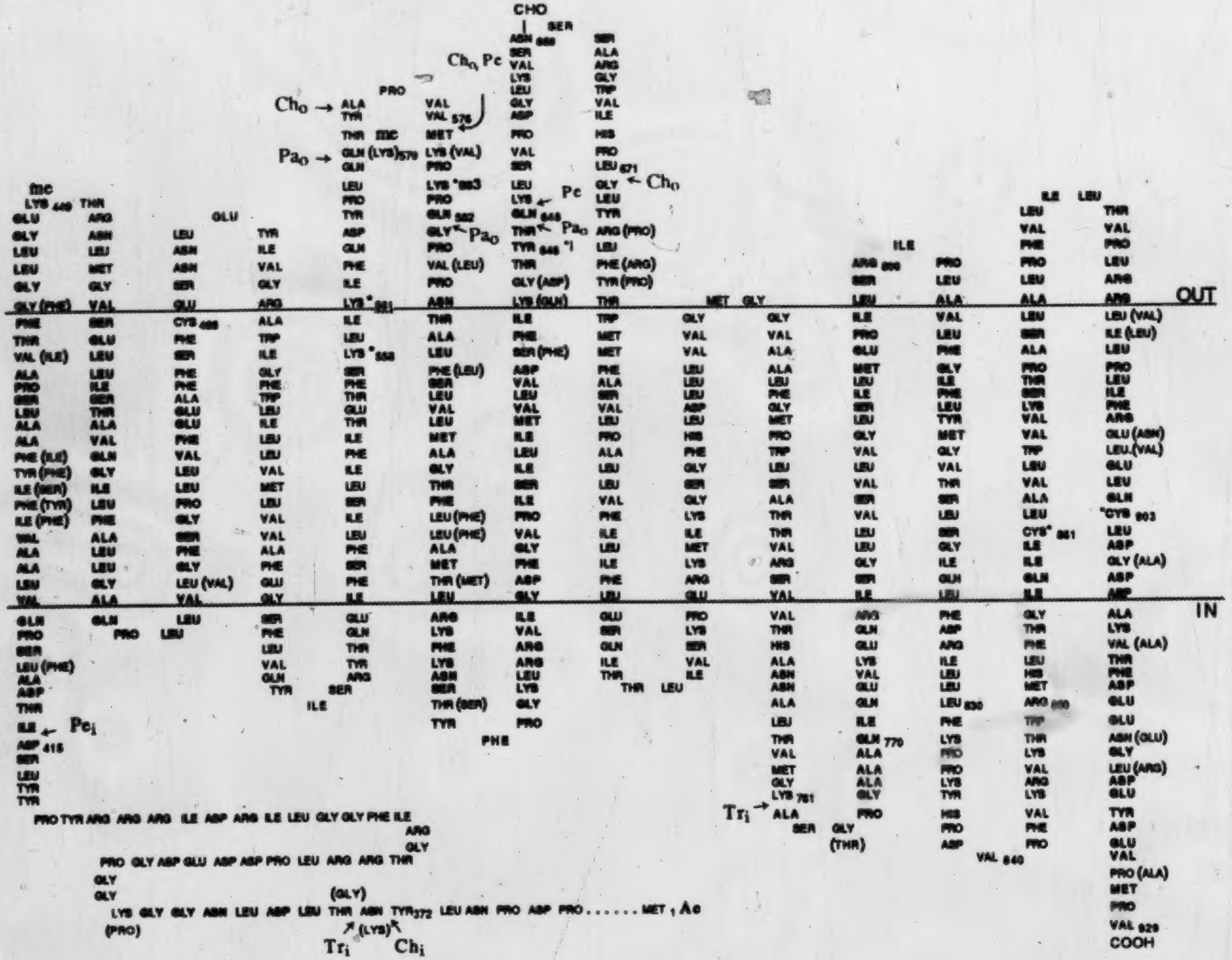

N
C

murine Band 3 sequence. The human sequences align without requiring many insertions or deletions, and the characterized sites of cleavage of the human protein are in most cases readily identified in the murine sequence (for details on the comparisons and alignments refer to: Kopito and Lodish, 1985a,b; Jay and Cantley, 1986). The homology between the deduced murine sequence and the portions of the human protein sequence is excellent for the portions from the membrane domain, but is lower for the sequences of the N-terminal segment (NCn-22) than for the C-terminal sequences (Kopito and Lodish, 1985a). Despite the striking homology in certain sections of the sequence, however, there are certainly differences between the murine and human sequences, for example in the content of histidine residues (see Jay and Cantley, 1986), and cysteine residues (see Chapter VI). This emphasizes that the murine sequence must be used with caution as a substitute for the human sequence when integrating the existing structural information for the human protein with the murine sequence. Two potential sites of N-linked glycosylation (-Asn-X-Ser/Thr) were identified in the murine sequence (Kopito and Lodish, 1985a). The location of the single site of glycosylation in the human protein has been located by an end-labeling technique to a region corresponding to the location at Asn-660 (Jay, 1986). Hereafter, when a site or residue from the sequence is denoted by number, the number refers to the residue number in the corresponding sequence of the murine protein.

3. Model of the Folding in the Membrane

We present here a model for the folding of the Band 3 polypeptide in the membrane (Figure I.3). The sequence of the C-terminal portion of murine Band 3 is shown, but those residues which have been confirmed in the human protein sequence are denoted in boldface, and any known differences in the human sequence are indicated in parentheses. This model has been developed on the basis of computer analysis of the murine sequence for hydrophobic sequences (Kyte and Doolittle, 1982), and for predicted turns (Chou and Fasman, 1978) (both performed by Dr. M.Ohnishi), and the known sites of vectorial

Figure I.3 A model for the folding of Band 3 protein through the erythrocyte membrane.
Only the sequence for the membrane domain (the C-terminal 55 kDa) is shown. The sequence and subscripts denoting residue numbers are those of murine Band 3. Those parts for which the sequence of the human form has been determined (by protein sequencing methods) are noted: marked in boldface if identical, and if different, the residue found in human is noted in parentheses. The folding arrangement is based on both the predictions for hydropathy and turns in the murine sequence, and the known sites of vectorial chemical labeling or proteolysis. Abbreviations are: (Ac) acetylated N-terminus; Ch, chymotrypsin cleavage site; Tr, trypsin cleavage site; Pa, papain cleavage site; Pe, pepsin cleavage site; subscript $_i$ denotes in, and subscript $_o$ denotes out; *I, iodination site; me, site of reductive methylation; *BS^3 alternate BSSS crosslinking site; LYS*-561 and -558 are potential DIDS-binding residues. There are several other sites of labeling that are not depicted in this figure (see Knauf, 1986).


CHO
OUT
IN
Cho
Cho, Pc
Pao
Pc
Pei
Tri
Chi
COOH
MET, Ac

chemical labeling and proteolysis (Jay and Cantley, 1986; Knauf, 1986). Two very similar models for the arrangement of the membrane domain of Band 3 in the membrane have been proposed, one containing 10 (Jay and Cantley, 1986), and the other containing 12 transmembrane crossings (Kopito and Lodish, 1985b). The model presented here agrees with the major features of the earlier models, but includes 14 transmembrane crossings. Additional experimental information is necessary to test such models and clearly define the folding and arrangement of Band 3 protein in the membrane.

The rationale for the number of transmembrane crossings in our model will be reviewed. TrCh-17 probably crosses the membrane a minimum of three times, based on the constraints of the protein chemical data: the N-terminus is the intracellular trypsin (or chymotrypsin) site, Lys-449 is an external site of methylation (Jennings and Nicknish, 1984), and the C-terminal chymotrypsin site (Jennings and Adams, 1981; Brock *et al.*, 1983) (this necessitates an odd number, but requires only one span). It has also been suggested that degradation of this DIDS-labeled fragment appears to generate three 4 kDa segments, one of which is labeled with DIDS (Ramjeesingh, Gaarn and Rothstein, 1984). Our model suggests there could be as many as 5 transmembrane crossings in TrCh-17, based on hydrophobicity, the predicted turns, and the lack of extracellular sites of proteolysis within this fragment. If there were fewer than 5 spans a substantial amount of the protein would be outside the membrane and would be expected to be accessible to proteases. This does not appear to be the case, suggesting that the bulk of the polypeptide is within the membrane. This model would require some charged residues to be in transmembrane segments, and would place Cys-498 on the extracellular side of the membrane, perhaps near the bilayer interface. The accessibility of this cysteine residue is addressed in Chapter VI of this thesis.

Within ChC-35, the evidence for the number of transmembrane crossings is less definitive than for TrCh-17. There is strong evidence for the N-terminal end of this fragment, corresponding to ChPa-8, having two crossings, knowing that the papain cleavage sites at each end are outside, and that Tyr-614 is not iodinated from the outside (Brock *et al.*, 1983). From this point to the C-terminus, the constraints on the folding are: the extracellular location of the papain and glycosylation sites, an internal trypsin-sensitive site that corresponds to Lys-761/Ala (Jennings, Anderson and Monaghan, 1986), a proposed intracellular location for the pair of cysteine residues, Cys-861 and Cys-903 (Rao, 1979), and the intracellular location of the C-terminus (Lieberman, Nattriss and Reithmeier, 1987; Lieberman and Reithmeier, 1988). Thus, there must be an odd number of crossings in PaC-28. The hydrophobicity analysis indicates the possibility of 5 transmembrane crossings. Our model suggests seven, but it is possible to form models having 3 or 5 transmembrane crossings that are consistent with the four constraints. Support for many membrane spanning segments comes from the heavy labeling of ChC-35 by hydrophobic labeling reagents (Guidotti, 1980; Brunner and Semenza, 1981), and the paucity of protease-sensitive sites exposed in situ. This model, and the one with 5 crossings, also places the pair of cysteine residues in this fragment on adjacent helices, buried in the hydrophobic region of the membrane -- a location that might be used to rationalize their proposed crosslinking and NEM-unreactivity upon formation of ghost membranes (Rao, 1979).

4. Secondary Structure

In the absence of a crystal structure, we will have to rely on spectroscopic techniques for information on the secondary structure of Band 3 protein. Most of the conformational studies on Band 3 protein have used the technique of circular dichroism, which is one of the most sensitive measures of the conformation of proteins in solution. The absorption of circularly polarized light by the peptide backbone is very sensitive to the peptide geometry, such that characteristic spectra are produced by the different types of protein secondary structure (Adler *et al.*, 1973). Empirical methods for obtaining estimates of the average secondary structure of a protein from CD data have been developed (e.g., Chen, Yang and Martinez, 1972; Chang *et al.*, 1978). Analyses of the far ultraviolet CD spectra of Band 3 support the idea that Band 3 protein contains a substantial amount of α-helical structure.

a. Circular Dichroism Measurements on Erythrocyte Membranes. The earlier reports of circular dichroism studies on this system were on isolated erythrocyte membranes (Lenard and Singer, 1966; Schneider *et al.*, 1970; Glaser and Singer, 1971; Urry *et al.*, 1971; Gordon and Holzwarth, 1971; Holzwarth, 1972). These reports represent pioneering efforts at application of circular dichroism to study membrane preparations. The CD spectra of the membranes showed some of the characteristic features of samples containing α-helical structure, but they also contained certain anomalous features: a lower than usual ratio of $[\theta]_{192} / [\theta]_{222}$, and a red-shift in the spectra above 220 nm, causing a shift to longer wavelengths of about 1 to 3 nm in the ellipticity minima near 222 nm (Glaser and Singer, 1971). These spectral anomalies seemed to be characteristic for several kinds of membranes (Lenard and Singer, 1966), and are thought to have their origin in the particulate nature of the samples, rather than some peculiarity of membrane protein conformation. Although it is thought by some that the presence of such artifacts make the spectra uninterpretable without major corrections (Urry *et al.*, 1971), it has also been shown that the value of $[\theta]_{222}$ is relatively insensitive to the optical artifacts, and can be used to obtain a reasonable estimate of the α-helical content (Glaser and Singer, 1971). The overall α-helical content of erythrocyte membrane proteins thus calculated is 40%. Of course, this represents an average of the content of all the proteins, of which Band 3 represents 25% by weight.

b. Spectral Artifacts. A discussion of the origin of these spectral artifacts in CD spectra of membrane samples is relevant here, since the presence of spectral artifacts will be considered in Chapter III. Several treatises on the origin of, and on the practical treatment of data containing, these spectral artifacts exist (Urry and Ji, 1968; Gordon and Holzwarth, 1971; Schneider, 1973, and references therein; Long and Urry, 1981, and references therein). One source of spectral artifacts is *light scattering*, which results from the large size of the membrane particles relative to the wavelength of the light. The dispersion of incident light in directions other than the forward direction results in fewer photons being detected as transmitted, thus, producing a higher apparent absorption. One effect on the unpolarized scattering is a reduction in total intensity of the signal. *Differential light scattering* can also occur if the refractive indices for left and right circularly polarized light are different. The net sign and magnitude of the *differential* scattering is a function of several parameters of the particle geometry, and is not easily calculated. Techniques for instrumentally eliminating the differential scattering effects have been developed, by changing the detector geometry (Bustamante *et al.*, 1983) or using fluorescence-detected CD (Reich *et al.*, 1980).

A more significant spectral artifact that is observed in membrane preparations is *differential absorption flattening*, which results from inhomogeneous distribution of the membrane proteins throughout the sample. This phenomenon was first described by Duysens (1956) and is manifest as a reduction, or flattening, of the apparent absorption of a suspension of particles at high concentration. It can be conceptualized as resulting from a confinement of the absorbing particles to a smaller physical cross-sectional area than if they were uniformly dispersed, reducing the probability of light encountering a chromophore, thus reducing the absorption; even samples containing uniformly dispersed membrane particles, each with randomly distributed proteins would be subject to this effect (Wallace and Mao, 1985). This effect is maximal at the peak of absorption, and is observed to different extents in both the unpolarized and the polarized absorption by particulate samples. The effects can be calculated and described by an absorption flattening coefficient, which is a function of particle size, shape, and absorptivity within the particle (Gordon and Holzwarth, 1971). Exemplary work in the area of minimizing, and correcting for, the absorption flattening artifacts in membrane samples has been provided by Wallace and co-workers in their study of bacteriorhodopsin (Wallace and Mao, 1984; Mao and

Wallace, 1984; Wallace and Teeters, 1987). Their work indicates that both differential light scattering and absorption flattening can be minimized, at least for bacteriorhodopsin, when the protein is present in small unilamellar vesicles with high phospholipid to protein ratios.

c. Purified Band 3 in Detergent Solution. Several independent measurements of the CD spectrum of Band 3 purified in the presence of detergents have been made. Based on these measurements, the commonly accepted estimates of helical content of Band 3 are around 45 % α-helix. Comparison of the several spectra indicates the molar ellipticity at 208 nm is slightly greater than that at 222 nm, and both have values around -15,000 deg-cm^2-dmol^{-1}. None of these spectra measured values below 200 nm, so comparison of the ratio of $[\theta]_{192} / [\theta]_{222}$ cannot be made. In Ammonyx LO (dimethyldodecylamine oxide) the calculated α-helix is 43% (Yu and Steck, 1975a), while the values in $C_{12}E_8$ are 46% α-helix, 37% β sheet and 17% unordered (Oikawa, Lieberman and Reithmeier, 1985). The helix content was not reported for bovine Band 3 in $C_{12}E_9$ (Nakashima and Makino, 1980a,b), but the molar ellipticites indicated about the same content of α-helix as in the human protein.

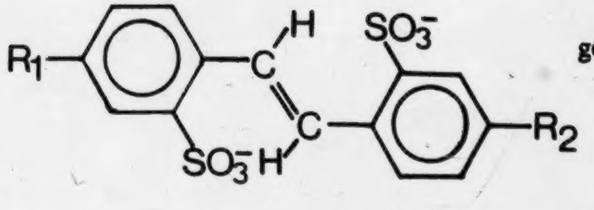
The CD measurements on detergent-solubilized protein are generally thought to represent the native conformation of the protein. Detergent-solubilized protein is, in fact, often regarded as a suitable reference state for physical studies, for example in the pseudo-reference-state approach to correcting CD spectra of membrane samples (Urry, 1972). Although nonionic detergents are usually considered to be non-denaturing, it is now apparent that the assumption that the structure of an integral membrane protein remains unaltered after solubilization may not hold true in all cases. Certain detergents have been shown to alter or fully disrupt the functional properties of membrane proteins (Werner and Reithmeier, 1985; Mao and Wallace, 1984 ; others are discussed in Chapter II). For this reason, it is important to establish that the detergent does not alter the protein structure, using independent measures of protein structural integrity and functionality.

Circular dichroism measurements have been used to detect changes in the secondary structure of Band 3, as in the measurement of denaturation by heat and chaotropic agents (Makino *et al.*, 1981; Oikawa *et al.*, 1985). Also, the influence of various detergents on the Band 3 protein structure can be monitored (Lieberman and Reithmeier, 1983). CD analysis of proteolytic fragments of Band 3 have also been done (Oikawa *et al.*, 1985), and indicate that the membrane domain is enriched in α-helix, with a content of 58%. The membrane-protected peptides remaining after extensive proteolytic digestion of membranes contained a very high α-helical content (86-94%), supporting the notion that the membrane-crossing segments are α-helical (Oikawa *et al.*, 1985).

5. Stilbenedisulfonate Binding Region and the Transport Site

A family of related compounds called stilbenedisulfonates have been used extensively in studying anion transport, both as potent, reversibly acting inhibitors of anion exchange, and as covalent labels (Knauf, 1979). The structures of some of these are shown in Figure I.4. These compounds inhibit transport only when added to cells from the external medium, indicating a binding site on Band 3 facing the outside of the cell (Shami *et al.*, 1978). Even with the derivatives that can react covalently, inhibition of transport can be produced by noncovalent binding alone (Shami *et al.*, 1978). Stilbenedisulfonates are competitive inhibitors of anion transport, as shown by the kinetics of inhibition (Shami *et al.*, 1978), and as shown by direct measurements of Cl^- binding using ^{35}Cl-NMR (Falke *et al.*, 1984a; 1985). Thus, the stilbenedisulfonate site seems to include the external anion binding site. An understanding of the nature of the stilbene site may also provide information as to the nature of the transport site, or anion binding site of Band 3. It is clear, though, that the stilbenedisulfonate binding site also includes other regions of the protein.

Structure-activity relationships of the inhibitory effects of stilbenedisulfonates and of classes of related inhibitors indicates that both electrostatic and hydrophobic interactions



general structure

R_1	R_2	abbreviation	name
-N=C=S	-N=C=S	DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonate
-N=C=S	$-NHCOCH_3$	SITS	4-acetamido-4'-isothiocyano stilbene 2,2'-disulfonate
$-NO_2$	$-NO_2$	DNDS	4,4'-dinitrostilbene-2,2'-disulfonate
$-NH_2$	-NHCO-⌬	BADS	4-benzamido-4'-aminostilbene-2,2'-disulfonate
S=C=N-⌬-CH_2-CH_2-⌬-N=C=S		H_2DIDS	1,2- (2,2'-disulfo-4,4'-diisothiocyano) diphenylethane (dihydroDIDS)

Figure I.4 Structures of stilbenedisulfonate inhibitors.
Only a few of the family of inhibitors differing in their substitution at the R_1 and R_2 positions of the stilbenedisulfonate nucleus are listed. The *trans* isomer is depicted in the general structure.

are important in making an inhibitor a potent one (Barzilay *et al.*, 1979; Knauf, 1979). The idea that the binding site is within a hydrophobic region is further supported by the observed enhancement of the fluorescence of stilbenes upon binding to Band 3. The quenching of intrinsic tryptophan fluorescence by stilbenes suggests there are tryptophan residues in or near the binding site (Rao *et al.*, 1979). Evidence for extensive interactions between the protein and inhibitor comes from the calorimetric and CD studies indicating that when DIDS is bound to the membrane domain the protein is stabilized to thermal denaturation by about 10 °C (Appel and Low, 1982; Oikawa *et al.*, 1985).

Important structural constraints on the requirements for the stilbenedisulfonate binding site are imposed by the evidence that the *trans* isomer of DNDS binds better than the *cis* isomer (Fröhlich and Gunn, 1980), and that the charged sulfonate groups appear to be neutralized by groups within the protein (Kampmann *et al.*, 1982). Derivatives with isothiocyanate substitutions also react covalently with Band 3 protein, with a particular reactive lysine residue in TrCh-17 that has been partially located in the sequence (Passow, 1986; see Chemical Modifications section). In addition, the bifunctional H_2DIDS can also react with a lysine residue in ChC-35 at pH values greater than 9, crosslinking the NCh-60 and ChC-35 proteolytic fragments (Jennings and Passow, 1979). The locations of these lysine residues spaced ≈16 Å apart should also be incorporated into the structural model of the stilbenedisulfonate binding site.

A relative location of the stilbenedisulfonate site as being below the outer surface of the membrane is supported by several independent distance measurements using resonance energy transfer between fluorescent probes (summarized by Knauf, 1986). For example, the distance between a fluorescent maleimide reacted with the cytoplasmic domain, and BIDS was found to be 34-42 Å, which is less than the width of the bilayer (Rao *et al.*, 1979). Also, bound eosin maleimide that had been reacted from the outside became accessible to quenching by Cs^+ from the intracellular solution, but not the extracellular solution, suggesting the site may extend some distance into the bilayer (Macara *et al.*, 1983). One current idea is that the stilbenedisulfonate binding site is in a hydrophobic region accessible through an aqueous cavity extending into the protein, below the surface of the lipid bilayer (Macara and Cantley, 1983). There is also a suggestion that the hydrophobic portions of the inhibitors may intercalate between hydrophobic contacts of adjacent helices (Knauf, 1986).

Synthesis of the information from several approaches leads to a generalized view of the stilbenedisulfonate binding site of Band 3 as being encorporated in a hydrophobic site below the surface of the bilayer that contains both two cationic residues (possibly arginines) in orientations to interact with the *trans* positions of the sulfonates, and two lysine residues in defined locations to be able to react with the isothiocyanate groups of H_2DIDS (see the models of: Passow, 1986; Macara and Cantley, 1983). Further definition of these specific amino acid residues in the protein sequence will be expected to better define the inhibitor binding site and transport site.

The specific structural requirements of the transport site, which is part of the stilbenedisulfonate site, have been studied by comparisons of structure and rates of transport of several substrates (Aubert and Motais, 1975). A recent piece of work using a series of phosphate analogs extends the earlier observations, and suggests that compounds with structural similarities to HCO_3^- are rapidly transported (Labotka and Omachi, 1987a). The development of a direct method of measurement of chloride binding using ^{35}Cl-NMR (Falke *et al.*, 1984a) makes possible the distinction between the requirements for chloride binding, as compared to stilbenedisulfonate binding. Although the binding of anions other than chloride cannot be directly measured using this method, the method has been used to classify the the effects of inhibitors as to their mode of action (Falke and Chan, 1986a-c), as discussed more fully in a following section.

D. Band 3 Protein Function

1. Functions Other than Anion Exchange

Band 3 carries out functions in addition to anion exchange (Low, 1986). A well-characterized function is that of providing the site of membrane attachment for cytoskeletal proteins. This is mediated by the cytoplasmic domain of Band 3, which contains a binding site for ankyrin, an extrinsic membrane protein which also interacts with spectrin, thus providing an anchor for the cytoskeletal network (Low, 1986). The association with the cytoskeleton also restricts the lateral mobility of Band 3. The binding of the β-chain of hemoglobin (Shaklai *et al.*, 1977) and glycolytic enzymes (Yu and Steck, 1975) to the cytoplasmic domain of human Band 3 is mediated primarily by the first 11 residues of the Band 3 polypeptide (Low *et al.*, 1984) A review by Low (1986) discusses the various structural features and functions of the cytoplasmic domain.

The covalently bound carbohydrate is thought to carry the blood group-specificity Rho-D antigen (Victoria *et al.*, 1987). Band 3 also is thought to be the senescent red cell antigen that signals the removal of aged or abnormal erythrocytes (Kay *et al.*, 1986; Winograd *et al.*, 1987).

A role of Band 3 in mediating the osmotic permeability of water and nonelectrolytes has also been suggested (Solomon *et al.*, 1983). Modification of a particular cysteine residue in Band 3 that is un-reactive to N-ethylmaleimide is thought to inhibit water and urea transport. Many inconsistencies in the pharmacologic, kinetic and radiation inactivation data cast doubt on the idea (Macey, 1984; Pitterich and Lawaczeck, 1985; Verkman *et al.*, 1986a,b; Jones and Fröhlich, 1984) -- for example, inhibitors of water transport have no effect on anion transport and vice versa (see Macey, 1984). Thus, it is now agreed upon by most workers that the water and urea permeabilites are not mediated by the same pathway as the anion transport, ruling out a common "aqueous pore". There is still some argument, however, for a role of regions of Band 3 separate from the anion transporting channel in allowing water permeation. The mode of mediation of osmotic water permeability through the erythrocyte membrane is still not known, and the apparently similar reactivities of the NEM-unreactive cysteine in Band 3 and the locus involved in water permeability remains a mystery.

2. Variations on the Function of Anion Exchange

In addition to catalyzing the exchange of monovalent anions, Band 3 protein also mediates the exchange of divalent anions. The evidence that this occurs through the same protein is that almost all of the inhibitors of Cl^- exchange also inhibit SO_4^{2-} exchange (Ku, Jennings and Passow, 1979). As will be described below, the pH-dependence of divalent anion exchange differs from that of monovalent anion exchange, which implies that there is a different mechanism. In fact, it is proposed that titration of the carrier protein with H^+ converts it from a form that preferentially transports monovalent anions to a form that transports divalent anions as well as monovalent anions (Gunn *et al.*, 1973). Additional evidence that the divalent anion mode of transport is carried out by a different form of the same carrier protein (that differs by the addition of a single proton) is provided by the observation that a proton is co-transported with sulfate, in exchange for chloride (Jennings, 1976). Because the protonation of SO_4^{2-} to HSO_4^- occurs at very low pH (pK_a=1.9), it is not likely that sulfate is itself protonated, rather, that a site on the protein is titrated by a proton. This mode seems to be a strictly coupled co-transport of anion and proton during sulfate transport, but the single proton binding site can be on the opposite side of the membrane from the anion binding site (uncoupled) during chloride transport (Milanick and Gunn, 1986).

An additional mode of anion transport that is mediated by Band 3 is that of net anion flux. A net Cl^- permeability has been measured (when the membrane is made permeable to K^+ by using an ionophore such as valinomycin) that occurs at a rate about 10,000 times slower than the Cl^- exchange (the original literature on this topic is well summarized by Knauf, 1986). A substantial fraction of the net anion flux (almost all, for SO_4^{2-}) is DIDS-sensitive. The net anion flux most likely does not occur by "slippage" of the unloaded transport site across the membrane, but instead, by movement of ions across the permeability barriers, without the usual conformational change in the carrier (also referred to as "tunneling") (Knauf, 1986). Evidence against a net flux occurring by a "slippage" mechanism is provided by, for example, the fact that at high sulfate concentrations, when the amount of unloaded carrier decreases, the net anion transport increases (Passow, 1977).

E. Mechanistic Aspects of Anion Exchange

In the following section further details of the anion exchange function will be presented, with emphasis on the relation of the function to structural features of Band 3 protein.

1. Kinetic Reaction Cycle. Kinetic studies have provided the basis for almost all the models of the mechanism of anion exchange. The kinetics of the system are similar to those predicted for a mobile carrier, while exhibiting a tightly coupled one-for-one exchange of anions (Knauf, 1979). However, since Band 3 protein is an integral membrane protein with multiple traverses of the bilayer, it has a defined asymmetry of orientation in the membrane and could not act as a mobile carrier. Two general mechanisms could result in such a tightly coupled exchange of anions. The first is a simultaneous mechanism (Sachs, 1977), in which two bound anions, one on either side of the membrane, are simultaneously translocated, providing a direct, mechanical explanation for the coupling. The second mechanism is a ping-pong mechanism (Cleland, 1963), in which a single anion binding site alternately faces inside and outside. As discussed below, data from several approaches, including kinetics, support the ping-pong mechanism.

The essentials of a ping-pong mechanism as applied to anion exchange can be described through an illustration of the reaction cycle, as shown in Equation I.1:

$$\begin{tikzcd} E_o \arrow[r, shift left] \arrow[d, shift right, "A_o"'] & E_i \arrow[l, shift left] \arrow[d, shift right] \\ E_oA_o \arrow[u, shift right] \arrow[r, shift left] & E_iA_i \arrow[l, shift left] \arrow[u, shift right, "A_i"'] \end{tikzcd} \qquad \ldots\ldots\ldots \text{[I-1]}$$

According to this model (as in Wieth, 1972, and in Knauf, 1979), an ion from one side of the membrane binds to a specific anion binding site, or transport site. The transport site and its bound anion are translocated across the permeability barrier such that they are exposed to the solution on the other side of the membrane. The anion can then dissociate from the binding site, leaving the site facing the internal solution. At this point a half-cycle of the exchange process has been completed. Anion binding, translocation, and dissociation in the opposite direction then completes the reaction of one cycle of anion exchange. A neccessary assumption in order for this model to describe the strict one-for-one exchange is that the transport site can be translocated only when a substrate anion is bound.

The evidence supporting this kinetic reaction cycle has been extensively reviewed (for example, Gunn and Fröhlich, 1979; Knauf, 1979; Wieth and Brahm, 1985; Passow, 1986; Knauf, 1986). Although Knauf (1986) is careful to point out that the kinetics cannot unequivocally distinguish between the two mechanisms, the data are generally recognized as being most compatible with a ping-pong mechanism, having a single anion binding site exposed on one side of the membrane at any given time. One approach to distinguish between these two mechanisms has been to measure the effect of gradients of anions (either different concentrations of the same anion, or anions with different transport rates) on the anion flux (e.g., Gunn and Fröhlich, 1979; Jennings, 1980). For example, when exchange of chloride for the more slowly transported sulfate is measured, the transport sites are apparently "recruited" to the side with sulfate. The sites on the chloride side fill and transport to the side with sulfate, but once filled with sulfate, return only very slowly to the chloride side (Jennings, 1980). There are also effects of chloride on the inside of cells on the binding of noncompetitive and competitive inhibitors on the outside of cells, specifically, an increase in potency in the presence of an outward chloride gradient (Knauf, 1986; Knauf and Mann, 1984). There are similar transmembrane effects of the binding of probes at one side on probes at the other side (Passow *et al.*, 1980b). Also, direct measurement of Cl^- binding by ^{35}Cl-NMR demonstrates both the effects of transmembrane ion gradients and reaction with inhibitors on recruitment of anion binding sites (Falke, Pace and Chan, 1984b; Falke and Chan, 1985). This evidence clearly indicates there is a transmembrane effect on transport, on chloride binding and on inhibitor binding, as predicted by the ping-pong model.

The most conclusive evidence for a ping-pong mechanism is the "half-turnover" experiment by Jennings (1982). When chloride-loaded resealed ghosts, with an amount of trapped chloride about three times the number of Band 3 molecules, were suspended in a medium of slowly penetrating anion, 1 x 10^6 chloride ions/cell were rapidly released, equal to the number of Band 3 monomers. This is thought to represent the half-turnover in the reaction cycle, and thus distinguishes the exchange from the simultaneous model (Jennings, 1985; Knauf, 1986). Some believe a version of a simultaneous model is still compatible with the above results, and better explains the interactions between ions observed for some divalent anions (Salhany and Rauenbuehler, 1983). There is no evidence for any simultaneous behavior in the transport of monovalent anions.

As illustrated by the reaction cycle, transport is composed of anion binding, translocation of the bound anion across the permeability barrier, and release of the anion on the opposite side. This distinction potentially allows the resolution of the effect of different amino acid residues on the different parts of the reaction cycle, and to similarly locate the effects of inhibitors. One very important finding in this respect was that the dramatic differences in the rates of transport for different anions does not correlate with the order of their substrate affinities (Dalmark, 1976; Knauf, 1979). To illustrate, the K_s for phosphate and Cl^- are similar, but phosphate is transported over 10^4 times slower than Cl^-. This suggests that the anion binding affinity does not determine the rate of transport. Therefore, the translocation step must be rate-limiting in the overall process, and the nature of the anion bound to the transport site would be expected to influence this translocation rate.

Another important development in studying the different steps in the reaction cycle is the use of ^{35}Cl -NMR to directly measure chloride binding to the transport site of Band 3 (Shami *et al.*, 1977; Falke *et al.*, 1984a). This allows observation of effects limited to substrate binding, allowing a method for experimentally separating the factors determining anion binding from those influencing the translocation. Direct binding measurements have reproduced some of the major kinetic findings, including transmembrane recruitment of anion binding sites (Falke *et al.*, 1984b; Falke and Chan, 1985), and the relative rates of the binding and translocation steps (Falke *et al.*, 1985a). A vestigial anion binding site has been located in the transmembrane segments that are protected from extensive papain digestion (Falke *et al.*, 1985b). Several anion transport inhibitors have been classified as to the part of the reaction with which they interfere (Falke and Chan, 1986a-c): using differences in the effect of Cl^- concentration on the binding of each inhibitor, the inhibitors were grouped as transport site, channel-blocking, or translocation inhibitors.

The ability to measure substrate anion binding directly is important, since inhibitor binding is different from substrate binding. For those inhibitors that have been shown to competitively inhibit transport, or whose binding is competitively inhibited by substrate anions, the anion binding site is expected to comprise a part of the inhibitor binding site, in the absence of allosteric effects. Because most inhibitors are larger than the preferred substrates, there are likely to be more amino acid residues in the protein that are involved in the interaction with a particular ligand or inhibitor, than with a substrate (Knauf, 1986).

An intrinsic asymmetry in the orientation of unloaded transport sites has been determined (Gunn and Fröhlich, 1979), indicating there are about 15 times the number of inward-facing transport sites as outward-facing when $Cl_{in}=Cl_{out}$. There appears to be no substantial difference between the Cl^- dissociation constants at the inside and the outside, as determined from the Cl^- effect on the inhibitory potency of niflumic acid (Knauf and Mann, 1984). This also means the asymmetry of the loaded transport sites is the same as the unloaded sites. The asymmetry must then originate from the differences between the translocation rate constants, with the outward rate constant being about 15 times smaller than the inward rate constant. For sulfate, there is a similar asymmetry, and it is pH-dependent (Jennings, 1980), but the rate constants for Br^- are symmetric (Gunn and Fröhlich, 1979). It is not known whether the asymmetry has any functional significance, or is just a consequence of the different free energies of the two states of the protein (Knauf, 1986).

2. Transport-related Conformations. The ping-pong model predicts, and recruitment of the anion transport site further implicates, that a conformational change in the Band 3 transport domain is associated with anion exchange. Although the exact molecular details of the conformational changes are unknown, two conformations of the transporter can be distinguished that are thought to correspond to the inward-facing and the outward-facing transporter. A major part of the evidence for these two conformations is based on differences in the reactivity of chemical reagents with Band 3 protein when it is treated under anion gradients expected to recruit the transport sites. For example, the rate of dinitrophenylation of the H_2DIDS-reactive lysine residue (Lys *a*) is reduced significantly by intracellular 2-(4'-aminophenyl)-6-methylbenzene thiazole-3'-7-disulfonate (APMB) binding at the internal transport site (Passow *et al.*, 1980). In an example of opposite orientation, DNDS or *p*-nitrobenzenesulfonate, which competetively inhibit Cl^- exchange by binding at the external side of the membrane, inhibit Cl^- binding to the cytoplasmic side of the membrane (Falke, Pace and Chan, 1984b). Many more examples exist, and are well covered in the reviews by Passow (1986) and Knauf (1986).

Simply the binding of substrates to the transport site is sufficient to cause similar changes in exposure and reactivity of amino acid residues in Band 3, as detected by changes in reaction rate of lysine with fluorodinitrobenzene (Passow *et al.*, 1980a; Passow *et al.*, 1980b), and on the signals of electron paramagnetic resonance probes (Ginsburg, O'Connor and Grisham, 1981). The more important conformational changes, however, would be those specifically associated with the translocation of the anion transport site with bound anion. Measurements suggestive of a transport-related conformational change have been done using large fluorescent inhibitors of anion exchange. Using fast kinetic techniques, the noncovalent binding of the fluorescent stilbenedisulfonate DBDS was resolved into two components: a rapid binding step, followed by a slow "conformational change" that may represent a partial translocation of the bulky anion (Dix *et al.*, 1979; Verkman *et al.*, 1983). Similarly, the fluorescence of eosin maleimide, which had been covalently reacted with the transport site from the external medium, was quenched by Cs^+ added from the cytoplasmic side, but not from the external side (Macara, Kuo and Cantley, 1983). The authors' interpretation was that the eosin maleimide had been partially translocated and thus made accessible to the internal medium. The additional observation of an altered exposure (accessibility to quenching by Cs^+) of tryptophan residues in the cytoplasmic domain further supports the idea of a conformational change in Band 3 protein upon binding certain inhibitors, but it is not clear that this is representative of the translocation step in transport.

Because of the necessity of rapidity of any conformational change associated with transport (10^5 events$\cdot$sec$^{-1}\cdot$Band 3^{-1} for Cl^- at 37 °C) it is suggested (Knauf, 1986) that it involves movement of only a small portion of the protein. In support of this, the far ultraviolet circular dichroism spectrum of Band 3 was not appreciably changed by the addition of DIDS (Oikawa, Lieberman and Reithmeier, 1985), indicating the absence of any significant rearrangement of the existing secondary structure. Yet the evidence above indicates that the change upon translocation of inhibitors is not restricted to only a very few residues, since it can be monitored by allosteric changes in the conformation (reactivity or exposure) of sites that are distinct from the transport site.

3. pH Dependence of Transport. The pH dependences of the different modes of exchange fluxes mediated by Band 3 formed the basis of the titratable carrier model (Gunn, 1972) of anion exchange. The pK_as of the titratable residues that affect anion exchange also provide insight as to the classes of amino acid residues that are involved in exchange. The plot of the exchange flux of Cl^- *versus* pH indicates that the flux increases with increasing pH at pH > 5.5, and shows a broad, flat maximum between pH 7 and 10, when measurements are done in resealed ghosts (Funder and Wieth, 1966; Wieth *et al.*, 1982a). The decrease at higher pHs occurs at pH 8 when measurements are done in cells, and is attributed to a decrease in intracellular Cl^- concentrations due to the increased negative charge on hemoglobin (Funder and Wieth, 1966). In resealed ghosts a slight increase in the rate is sometimes observed between pH 10 and 11, and the transport is inhibited above pH 11, by titration of a group of $pK_a \approx 12$ (Wieth *et al.*, 1982a).

The pH dependence of sulfate transport is distinctly different from that of Cl^- exchange, demonstrating a much narrower pH range that produces maximal transport rates (Schnell *et al.*, 1977). As pH is decreased from 7.4 to 6.5 sulfate flux increases, while Cl^- exchange was shown to decrease over this range. The pH maximum is around pH 6.2, with apparent pK_as of the activating and inactivating protonation sites of about 6 and 7, respectively. The transport of phosphate (Schnell *et al.*, 1981) has a pH-dependence very similar to that of sulfate. Interpretation of this result must be cautious, since phosphate is a readily titratable anion ($pK_a = 6.8$), unlike sulfate ($pK_{a2} \approx 1.9$). It is possible that divalent HPO_4^{2-} is cotransported with a proton, similarly to sulfate. Evidence that the monoanionic form of phosphate ($H_2PO_4^-$) is the actively transported form (Brauer *et al.*, 1985), and the possibility of simulating the experimental pH dependence by an overlap of the Cl^- pH-dependence with the titration of $H_2PO_4^-$ to HPO_4^{2-} (Brauer *et al.*, 1985) suggests phosphate may be transported chiefly by the Cl^--transporting form of the transport protein.

The alkaline branch of the pH dependence of Cl^- transport has been studied thoroughly by Wieth and Bjerrum (1982). Two sets of groups titrate in this region, and both are thought to represent arginine residues because of the high pK_as. One functional group deprotonates with an apparent external $pK_a \approx 11$, resulting in a slight stimulation of transport, if the measurements are done at high Cl^- concentration. This titratable group apparently may represent the halide-binding, inhibitory "modifier" site proposed by Dalmark (1976). The apparent stimulation of flux between about pH 10 and 11 would then be explained as release of the inhibition by this site. Deprotonation of the other site, with a $pK_a \approx 12$ (at external concentrations of Cl^- of 0.1 M), results in inactivation of Cl^- exchange, and has been thought to represent titration of the transport site residue(s). The pK_a of this site is sensitive to Cl^- concentration (at low concentrations), and does not appear to be affected by the orientation of the transporter (Wieth and Bjerrum, 1982). These results indicate that the titratable site does not seem to be the actual site of anion binding, but may be a site adjacent to the anion binding site that is also necessary for transport (Knauf, 1986). In addition, the pK_a of the specific amino acid residue that corresponds to Lys *b* has been measured by monitoring the pH dependence of crosslinking after reaction of one end of H_2DIDS with Lys *a* (Kampmann *et al.*, 1982). By this method, Lys *b* has a pK_a of 10.0, and a rough estimate of the pK_a of Lys *a* is between 7 and 8. The crosslinking reaction was carried out at 37 °C, and the pK_as of these residues would likely be substantially higher at the temperatures (0-4 °C) of measurement of Cl^- exchange. Thus, it is not clear whether these residues correspond to the two alkaline branch residues whose titration affects transport.

The acidic branch of the titration appears to involve several different titrating residues. By separately varying either intracellular or extracellular pH, the protons titrating from sites that are either on the inside or the outside of the membrane, respectively, can be distinguished. Several reports indicate the presence of a group that titrates from the external media with a $pK_a \approx 5$ which, when protonated, results in inhibition of Cl^- exchange (Wieth *et al.*, 1980; Wieth *et al.*, 1982a; Milanick and Gunn, 1982; Milanick and Gunn, 1986). The binding of protons to an intracellular site with a $pK_a \sim 6.1$ also acts to inhibit Cl^- exchange (Wieth *et al.*, 1980; Milanick and Gunn, 1986). Milanick and Gunn's results indicate that protonation of a group with $pK_a \sim 5.5$ (at 0 °C) on the outside activates Band 3-mediated sulfate influx. They proposed (Milanick and Gunn, 1984) that this sulfate transport-activating protonation site could be the same site as the site of H^+ inhibition of Cl^- exchange, based on the similar pK_a (Cl^- inhibitory site $pK_a \sim 5$ at 0 °C). Thus, the internal and external Cl^- transport-inhibitory pK_as were suggested (Milanick and Gunn, 1986) to be due to a single site with pK_a that varies depending on its exposure, and the proton of this site was suggested (Milanick and Gunn, 1986) to be the proton that is cotransported with sulfate (Jennings, 1976, 1982a). A titration that may be distinct from the sulfate transport-activating proton involves an intracellular site that causes inhibition of sulfate self-exchange when it is protonated below pH 6 (lowering only extracellular pH does not titrate it) (Schnell *et al.*,1977; Milanick and Gunn, 1984). Although most of the reports cited imply that the titrating residues in this pH range are titrating from neutral to negatively charged species, there is an alternate interpretation, which is proposed in one report (Labotka and Omachi, 1987b), that for some of the titration effects in this pH region the titration may be of a positively charged to a neutral species.

Two observations relating to the role of these protonatable residues in anion exchange both indicate that the residues titrating in the region of pH 6.0 to 9.0 are not essential for substrate anion binding, but must somehow be required for the translocation step of catalysis. The apparent half-saturation constants of chloride and sulfate decrease slightly as pH is elevated, and that of phosphate is not susceptible to changes in pH (original references are summarized by Kaufman, Eberl and Schnell, 1986). Nor is the binding affinity of NDS-Tempo (N-4-(2,2,6,6-tetramethyl-1-oxyl)piperidinyl-N'-4-(4'-nitro-2,2'-disulfonatostilbene)thiourea), which has been shown to compete for both chloride and sulfate binding, dependent on pH between pH 6.0 to 8.0 (Kaufman, Eberl and Schnell, 1986).

4. Residues Involved in Anion Exchange and Inhibitor Binding

The titration of amino acid side chain groups that influence anion exchange fluxes has provided an introduction to the classes of residues that are implicated to have a role in exchange. Substantial evidence for functions of specific amino acids or types of amino acids has also come from studies using inhibitors of anion exchange, and group-specific modifying reagents. The approach of using group-specific or site-directed chemical modification to identify the proteins involved in a particular function, or to identify functional amino acid residues within a protein, is a classic one, having been applied in numerous instances to soluble proteins and resulting in an abundance of literature on the subject (see, e.g., Means and Feeney, 1971; Glazer, Delange and Sigman, 1975; Hirs, ed., 1967; Hirs and Timasheff, eds., 1972). The first attempt at using chemical modification to relate inhibition of anion transport in erythrocytes to modification of membrane proteins was in 1952 (Edelberg, 1952). Extremely important information has been obtained from similar studies, with the major finding of course being the identification of Band 3 protein as the anion exchange protein (Cabantchik and Rothstein, 1974; Ho and Guidotti, 1975).

Further studies employing a variety of chemical modification reagents have been done to obtain direct information about the specific amino acid residues of Band 3 which may participate in binding and translocation of anions across the membrane. Several such sites of chemical modification have been at least partially localized in the Band 3 protein sequence. Other sites are shown to be modified, but do not affect anion transport function, and have been useful as vectorial labeling sites in helping to test models of the folding of Band 3 in the membrane.

Because in most of these studies the aim was to relate modification of a particular residue with some functional consequence, they usually have been performed on the native protein, by treating either intact erythrocytes or ghost membranes with the reagent. In this way, a measurement of transport function can be made directly, without complications arising from reconstitution. Performing chemical modification on a preparation of cells or sealed membranes provides another advantage -- the permeability barrier can be used to limit the accessibility of certain membrane-impermeant reagents to only those reactive groups on one side of the membrane. The potential disadvantages of doing chemical treatments on cells or membranes isolated from cells are obvious: there are many other potential reactive groups in the other components of the membrane that can provide contaminating modified groups from which the protein of interest must be isolated; there may also be concerns about a reagent disrupting the permeability barrier. The reaction with other proteins can be serious, since the possibility of losing a labile modification can be introduced during steps to isolate the protein. In addition, other reactive substances can compete for reaction with the reagent. For example, in NEM treatment of red cells, reactive groups on hemoglobin and glutathione compete for reaction with membrane proteins. Another disadvantage, which applies even to soluble proteins, is the lack of specificity of some modifying reagents.

Rather than taking a historical approach to summarize the chemical modification studies that have been done on Band 3 protein, I will summarize the results for each class of amino acid residue for which functionally relevant modifications have been made.

a. Arginine Residues. It is generally recognized that arginyl residues often provide their positively charged side chain as part of functionally important binding sites in several enzymes (Riordan, 1979). The anion binding site(s) in Band 3 should be no exception (Wieth *et al.*, 1982a). Two arginine-specific reagents, 1,2-cyclohexanedione and phenylglyoxal, have been shown to inhibit anion transport (Zaki, 1981; Wieth, Bjerrum and Borders, 1982; Zaki, 1983; Bjerrum, Wieth and Borders, 1983; Zaki, 1984; Zaki and Julien, 1985). 1,2-cyclohexanedione was first shown, by Zaki (1981), to inhibit anion transport, and to reduce the H_2DIDS binding by Band 3. The stoichiometry and location of reaction are unknown. A mechanism of transport inhibition by this modification that is distinct from modification of the transport site has been suggested, based on ^{35}Cl-NMR data (Falke and Chan, 1986b), in which the modification acts as a channel blocker, slowing the migration of Cl^- between the transport site and the medium.

The data for modification by phenylglyoxal is more extensive. Zaki finds that although the rate of inactivation by phenylglyoxal is pseudo-first order (Zaki and Julien, 1985), that incorporation of 4 to 6 molecules of phenylglyoxal, implying modification of 2 or 3 arginine residues, per Band 3 occurred under conditions producing almost complete inactivation (Cl^--free buffer, at pH 7.4, treating with 2.5 mM phenylglyoxal at 37 °C for 60 min) (Zaki, 1984). The [^{14}C]phenylglyoxal-labeled residues of Band 3 are located in both major chymotryptic fragments: two-thirds of the label is in the NCh-60, and about one-third is in the ChC-35 (Zaki, 1984). In contrast, the results from Wieth's group indicate the reaction of phenylglyoxal with ChC-35 with 1:1 stoichiometry is sufficient to produce maximum inhibition of anion transport (Bjerrum *et al.*, 1983). The 1:1 stoichiometry is found after reaction with phenylglyoxal at low extracellular KCl concentration, whereas a higher stoichiometry of labeling (presumed 2:1) accompanies the inhibition produced by treatment at high extracellular anions. Both treatment conditions employ a neutral interior pH and an alkaline exterior pH.

In red cells treated with phenylglyoxal under optimal conditions (Wieth's) the concentration of H_2DIDS, and time of reaction, required to achieve complete reaction was increased (Bjerrum *et al.*, 1983) and the reversible binding of DBDS was reduced in proportion to the inhibition of anion transport (Passow, 1986). This indicates that modification of the functionally essential arginine of ChC-35 significantly reduces the noncovalent stilbenedisulfonate binding affinity, significantly enough to hamper the covalent reaction. The Cl^- dependence of the pK of the modification reaction , and of the pH dependence of the transport process are similar, indicating that the phenylglyoxalated group is the same one that controls the pH-dependence of the anion transport at high pH (Wieth and Bjerrum, 1982). Direct evidence for the role of the modified arginine has been provided by the direct chloride binding measurements, although the conditions of phenylglyoxylation for these measurements are not the same as the others. Phenylglyoxylation eliminates the transport site ^{35}Cl-NMR linebroadening, and competes with Cl^- for binding (Falke and Chan, 1986a). Phenylglyoxal is thus an inhibitor that acts at the transport site.

b. Lysine Residues. Several lysine residues in Band 3 have been defined by their reactivity with inhibitors of anion exchange, and are classified by Passow (1986) as denoted with a suffix letter (e.g., Lys *a*). The first two of these have abnormally low pK_a values, and are unusually reactive to chemical modification. The lysine residue that has been defined by its reaction with the isothiocyanate derivatives of the stilbenedisulfonates H_2DIDS and DIDS is referred to as lysine *a*. Because the stilbenedisulfonates inhibit anion transport by noncovalent interaction alone (presumably by interaction of the sulfonates with the anion binding site residues) it is not possible to determine the functional role of Lys *a* using these inhibitors. However, H_2DIDS has been useful in localizing Lys *a* within the C-terminal third of TrCh-17 (Mawby and Findlay, 1982; Jennings *et al.*, 1986). The location to a peptide fragment within 7,000 to 9,000 daltons from the carboxyl-terminus of TrCh-17 (Grinstein *et al.*, 1978; Ramjeesingh *et al.*, 1980) is unlikely, since there are no lysine residues in the murine Band 3 sequence (Kopito and Lodish, 1985a) corresponding to this fragment. Lysine *a* likely corresponds to either lysine-558 or -561 of the murine sequence, however, the actual reactive residue, and the protein sequence around it in the human form, have yet to be determined.

Other amino-reactive reagents have reaction characteristics that indicate that they are reactive with the same lysine residue, the primary determinant being the demonstration of mutual exclusion of reaction, or prevention from reacting by the presence of bound stilbenedisulfonate. Dinitrophenylation of Lys *a* inhibits anion transport (Passow, 1978; Rudloff, Lepke and Passow, 1983), as does reaction with arylsulfonates such as isothiocyanobenzene sulfonate (Ho and Guidotti, 1975; Drickamer, 1976). Reductive methylation of Lys *a*, which maintains the positive charge of the side chain at neutral pH, does not inhibit transport (Jennings, 1982). Reaction with BSSS, which is thought to react bifunctionally with Lys *a* and a second lysine residue (lysine *b*, defined below) and neutralize their charge, inhibits transport (Jennings *et al.*, 1985). Thus, the charge on Lys *a*, or the other lysine, is important in transport function, but seems to be non-essential, since function can be restored at a lower pH (Jennings *et al.*, 1985). These modifications of Lys *a* are represented and described in more detail in Chapter V.

Lysine *b* is defined as the residue in ChC-35 that reacts with the second isothiocyanate of H_2DIDS and thus allows crosslinking of the ChC-35 to the NCh-60 fragment (Jennings, 1979). The location of Lys *b* has been defined by proteolysis and H_2DIDS crosslinking experiments to be within an NTCB subfragment of TrC-20 (Jennings *et al.*, 1986). The crosslinked product has a size consistent with its representing cleavage at Cys-861, limiting Lys *b* to between the trypsin site (Lys-761) and this Cys (Jennings *et al.*, 1986). The pK_a of Lys *b* has been measured by monitoring the pH dependence of crosslinking after reaction of one end of H_2DIDS with Lys *a* (Kampmann *et al.*, 1982). By this method, Lys *b* has a pK_a of 10.0, and a rough estimate of the pK_a of Lys *a* is between 7 and 8.

A lysine residue in ChC-35 is also reactive with dinitrofluorobenzene, but it is not identical to Lys *b*, since its reaction is not protected by stilbenedisulfonates, and it is denoted Lys *c* (Passow, 1986). It is allosterically linked to Lys *a*, and dinitrophenylation of Lys *c* enhances anion transport (Passow, 1986).

In addition to the amino-reactive stilbenedisulfonates, and reagents that are group-specific for lysine, studies using the lysine-reactive pyridoxal 5'-phosphate (PLP) have also been reported. PLP is a substrate of the anion transporter and can be thought of as a site-specific reagent, that can provide a covalently attached label after reduction of the Schiff base with tritiated sodium borohydride (Cabantchik *et al.*, 1975; Nanri *et al.*, 1983). The labeling of ChC-35, but not of NCh-60, is prevented by DNDS, and the modification of ChC-35 is responsible for the inhibition of transport (Nanri *et al*, 1983). A recent report suggests that the labeling of TrCh-17 by PLP is also inhibitory at high levels of incorporation (Salhany *et al.*, 1987). The labeling site in ChC-35 has been localized to PaC-28 (Matsuyama *et al.*, 1983) and, recently, to a hydrophobic peptide of $M_r = 5,300$ (Hamasaki *et al.*, 1987). This peptide has been sequenced, and aligns with residues 852 to 929 in the murine sequence, with the label identified in Lys-869 (Kawano *et al.*, 1988). It is interesting to speculate that the PLP-modified residue in ChC-35 may represent Lys *b*. The results of the H_2DIDS crosslinking (Jennings *et al.*, 1986) are more suggestive of a location for Lys *b* between murine residues 761 and 861, but are not inconsistent with a location between 761 and 903. Further experimentation is required to determine whether the site of PLP labeling in PaC-28 is equivalent to Lys *b*.

c. Carboxylate Residues The involvement of carboxyl residues in the anion exchange function was suggested by the demonstration that a titratable group of pK_a of 5 on the outside of cells is required for chloride exchange (Wieth and Bjerrum, 1982; Milanick and Gunn, 1982; Milanick and Gunn, 1986). Protonation of a group of similar pK_a was also shown to activate Band 3-mediated sulfate influx (Milanick and Gunn, 1984). This pK_a would normally suggest a histidine residue, but since histidine has a large enthalpy of ionization, the lack of temperature dependence of the pK_a in this case suggested a carboxylate (Wieth *et al.*, 1980; Wieth *et al.*, 1982a).

The postulated involvement of carboxyl residues is supported by chemical modification studies, using carbodiimides as carboxyl-selective reagents to inhibit anion transport (Deuticke, 1977; Wieth, *et al.*, 1982a,b; Andersen *et al.*, 1983; Bjerrum, 1983; Craik and Reithmeier, 1984; Craik and Reithmeier, 1985). Using the membrane-impermeant carbodiimide ETC, two laboratories have demonstrated inhibition of anion transport. Craik and Reithmeier (1985) indicated that the characteristics of the inhibition produced appeared to be sensitive to the reaction medium, being reversible in one case, and irreversible in another. There was no incorporation of radiolabeled carbodiimide, and addition of glycine ethyl ester had no effect on the reversible inhibition. The differing inhibition characteristics were proposed to be due to the difference in orientation of the anion binding site of the protein in the two reaction media (Craik and Reithmeier, 1985). However, it was not determined which residues are modified or what accounts for the difference in transport inhibition in the two conditions. This topic is addressed in Chapters IV and V of this thesis.

Wieth's group reported (Wieth *et al.*, 1982a) a readily produced inactivation of anion transport by 50%, and stable inhibition under all conditions after treatment of resealed ghosts with ETC. The inhibition was accompanied by a reduction in the number of DIDS molecules required to further inhibit transport (Wieth *et al.*, 1982a), suggesting that ETC had inhibited activity in 50% of the transporters. They also found an accelerated rate of inactivation by addition of tyrosine ethyl ester (Andersen *et al.*, 1983; Bjerrum, 1983), and reported incorporation of radiolabeled tyrosine ethyl ester into Band 3, into the ChPa-8 fragment (although no evidence was shown) (Bjerrum, 1983).

Using another carboxyl reagent, the isoxazolium based Woodward's Reagent K, Jennings and Anderson (1987) recently provided evidence that modification of at least two glutamate residues in Band 3 results in transport inhibition. Two sites of less than stoichiometric labeling were detected, one being located in TrCh-17, and one in ChC-35. Both sites were within the stilbenedisulfonate binding region. The ability to produce the reduced carbinol form of the modified glutamate residues provides the possibility for interesting functional studies. Recent results characterizing the function of the transporter with the carbinol form of the glutamate in ChC-35 indicate that this glutamate provides the titratable site that binds and cotransports a proton with sulfate (Jennings, 1988; Jennings, personal communication).

d. Histidine Residues. Despite the suggestion that a residue of positive charge with a $pK_a \cong 6$ may be contributing to the pH dependence of anion transport (Labotka and Omachi, 1987b), little work has been published on histidine modification in Band 3 protein. Based on the pH-dependence of transport, an intracellularly located histidine residue has been suggested to be important for the transport of PLP and P_i (Matsuyama *et al.*, 1986). Studies of the binding of eosin-5-isothiocyanate (EITC) to Band 3 have also suggested there are functionally important histidine residues. EITC is known to be an inhibitor of sulfate exchange in erythrocytes (Knauf and Mann, 1984), and is suspected to bind to a modifier site on Band 3 (Sato *et al.*, 1986). Measurements of the pH-dependence of the absorbance of EITC covalently bound to Band 3 showed titrations around pH 6.4 and 13.1 that affect the binding of EITC (Chiba *et al.*, 1986) In addition, the EITC-reacted ghosts have an induced CD band at 520 nm due to bound EITC, which is reduced by the histidine reagents diethylpyrocarbonate ($I_{50} \cong 1\ \mu M$) and p-diazobenzene sulfonate ($I_{50} \cong 80\ \mu M$) (Chiba *et al.*, 1987). Diethylpyrocarbonate also inhibits anion transport, but requires higher concentration ($K_i \cong 2$ mM). The pH-dependence of the CD intensity change induced by EITC corresponds to that of the inhibitory effect (Chiba *et al.*, 1987). These results, especially the inhibition of transport by treatment with histidine reagents, provide very suggestive support for a role of histidine residues in anion transport.

e. Cysteine Residues. Despite a small amount of evidence to the contrary, it is generally agreed that none of the cysteine residues in Band 3 is involved in the anion transport function. Treatment with NEM, which results in modification of all but one cysteine residue in Band 3 (Rao, 1979), or with organic mercurials, does not inhibit anion transport (Knauf and Rothstein, 1971). Deuticke (1982) reported that NEM does inhibit anion transport, but suggested this effect was a result of modification of an amino group, rather than of a cysteine. However, another sulfhydryl reagent, diamide, was found to inhibit anion transport (Deuticke, 1986). DTNB has been shown to inhibit anion transport, but this effect is due to noncovalent binding, not due to reaction with sulfhydryls (Reithmeier, 1983). Others have shown that neither pCMBS nor DTNB treatment inhibited net chloride flux (Knauf *et al.*, 1983). The controversy of modification of cysteine residues of Band 3 in affecting the osmotic water permeability in erythrocytes was covered in an earlier section, and will also be examined in Chapter VI. In summary, the evidence suggests that there is not an essential role for any of the cysteine residues in the anion exchange function of Band 3.

5. Models of the Mechanism of Anion Exchange

Models of anion exchange have been developed primarily based on the evidence from kinetic studies. Any tenable model of anion exchange should be based on the ping-pong kinetics, predicting one-for-one exchange. It must also attempt to incorporate the following: the experimentally observed transport-related conformational changes (but with a fixed asymmetry of protein orientation); the effects on the translocation step of a) reagents binding outside the transport site, b) an unfilled transport site, c) the nature of the anion bound to the transport site; the effects of residues identified by chemical modification, as well as the titratable residues regulating monovalent and divalent anion transport; the information about the structure around the stilbenedisulfonate binding region and transport site.

A historical review of the models of anion exchange that have been proposed is provided by Passow (1986), who groups the models into two classes, "knock on" and "lock in", based on how the transported anion interacts with the titratable groups of the protein. In the "knock on" mechanisms the incoming anion breaks existing electrostatic interactions in the protein when it binds. The "lock in" models suggest entrapment of the anion by the anion binding residues. Both types have their basis in the titratable carrier model of Gunn (Gunn, 1972), and involve the formation of ion pairs between transported ions and charged amino acid residues in Band 3, and between amino acid residues in Band 3. More recently, another type of model has been proposed (Falke and Chan, 1986c) that is mechanically very different from previous models. It involves a segment of the protein forming a short hydrophobic α-helix that acts as a hydrophobic barrier, which would slide past charged residues located in a channel formed by a helical bundle.

The model presented here (Figure I.4) is adapted from that proposed by Knauf (1986), and represents a synthesis of some of the structural evidence into the functional model of the kinetic reaction cycle. It is an adaptation of the earlier "lock in" type of models (Passow, 1986; Passow *et al.*, 1980a; Knauf and Law, 1980), in suggesting that substrate anion binding in the region of two positive charges allows the charges to slip by one another. In addition, it also incorporates some of the features of the "knock on" type model (e.g., Macara and Cantley, 1983; Wieth *et al.*, 1982b; Brock, Tanner and Kempf, 1983) in incorporating anionic amino acid residues into the functional model. It includes

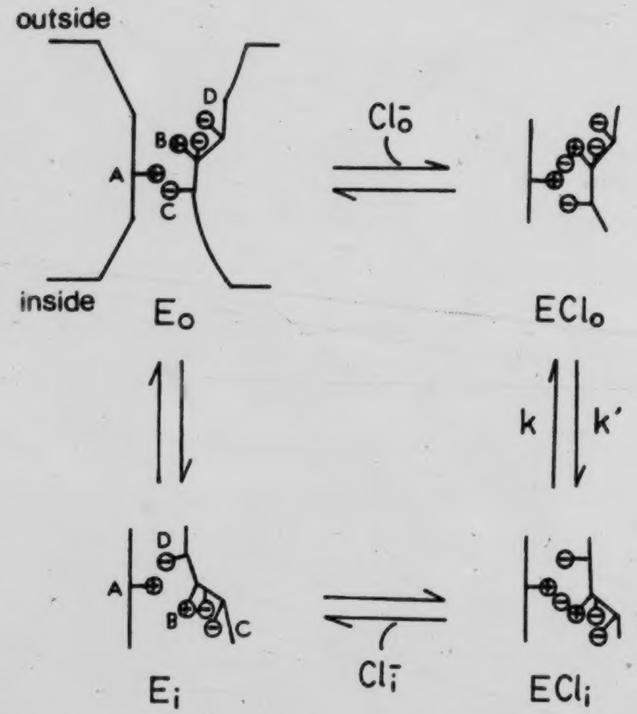


Figure I.5 Model of the mechanism of anion exchange and the kinetic reaction cycle. The anion transport site is depicted as being formed by two positive charges and two negative charges located within the interior of the folded transmembrane portion of Band 3 protein. Aqueous filled channels lie to either side of the transport site residues, and are thought to contain the "modifier sites" at which anions or inhibitors can act to modify the transport rate. The states of the transporter defined by the reaction cycle (Equation I.1) are depicted, with k and k' representing, respectively, the outward and inward rate constants for translocation of the loaded transporter. When the transport site is in the outward-facing form (E_o) anions from the outside can bind to the positive charges A and B, but anions from the inside are prevented from binding by the negative charge (C) blocking the positive charges. With an anion bound, the repulsion betweeen charges A and B would be reduced and allow a spontaneous conformational change between ECl_o and ECl_i, in which B moves past A. The transport site is then in the inward-facing form (E_i), in which charges A and B are accessible to cytoplasmic anions, and the binding of external anions is blocked by the negative charge D. It is assumed that the translocation of the transport site in either direction without a bound anion (interconversion of E_o and E_i) does not occur because of the repulsion between A and B. In this model net anion flux can be explained by assuming that the barriers formed by charges C and D may allow a finite diffusion of anions through the gate without the conformational change. We also depict a third negative charge located between the positive charge B, and the negative charge C, in such a way that it can be translocated across the permeability barrier with the bound anion. This is suggested to represent the anionic residue that is thought to be involved in proton cotransport with sulfate. A better location is required to account for the finding that this residue does not necessarily always change orientation each time the anion transport site does (Milanick and Gunn, 1986).

two negatively charged residues, one each at the inner and outer sides of the two cationic charges. The arrangement of the anionic residues is such that they would allow only a minimal diffusion of anions (low net flux, and an effective permeability barrier). They also prevent the binding of anions to the "wrong" side of the transporter, and are proposed to change conformation along with the anion-binding residues, such that the conformational change would be tightly coupled to the translocation of the bound anion.

There are three additional important aspects of this model. First, it also incorporates a provision for the experimentally demonstrated transport of divalent anions such as sulfate. The protonatable group that has been shown to allow this mode of transport (Milanick and Gunn, 1986) has been incorporated into the model in accordance with the suggestion by Knauf (1986) that it may lie between the outer and inner anionic residues to allow the H^+ to be co-transported during the conformational change. Note that this model also includes anionic residues in the protein for functions other than the binding of the proton that is cotransported with sulfate.

Second, the model suggests a structural feature -- aqueous channels, or funnels, separated by a permeability barrier (the exchange gate). The presence of an aqueous cavity is suggested by the relative location of the stilbenedisulfonate binding site within the bilayer (summarized earlier). There is also a suggestion that some inhibitors, while not competing with Cl^- for binding to the transport site, slow the access of anions to this site from the solution, which is consistent with their acting to block an aqueous access channel (Falke and Chan, 1986b). The lack of accomodation of such results was considered an inadequacy of the earlier "lock-in"-type models.

A third important feature of this model is that it does not imply that binding of an anion drives the translocation. In fact, Knauf stresses that anion binding (at least for Cl^-) does not *drive* the system toward either conformation, since binding does not change the relative free energies of the inward- and outward-facing forms of the transport site (Knauf and Mann, 1984). It is clarified in this model that anion binding acts to lower the activation free energy for the conformational change by reducing the repulsion between the two positive charges, allowing them to slip by one another at an appreciable rate (Knauf, 1986). The built-in constraint on translocation of an unloaded transport site would be the repulsion between these two positive charges when no anion is bound.

This model is based on the kinetic aspects of anion exchange, but includes some structural features of Band 3 protein as well. It is based on a ping-pong model, and adequately provides for a strict one-for-one exchange, a "barrier" to minimize the uncoupled diffusion of anions, and a recruitment effect of other anions and inhibitors. It also can accomodate divalent anion-proton cotransport, and includes titratable anionic residues other than the co-transported proton site. In addition, the transport site is buried in the membrane, inaccessible to proteases. The structural information about the stilbenedisulfonate binding site is also incorporated, proposing an "access channel" to the narrow transport "gate". It is also assumed that the "channel" is formed in some way by a bundle of the transmembrane α-helices. The more detailed structural models that have recently been proposed (Jay and Cantley, 1986; and Kopito and Lodish, 1985) include speculation as to the arrangement of the putative transmembrane helices in a bundle, and even of residues within this bundle that might interact. Though extremely interesting, these models should be regarded as premature until a definitive pattern of the protein's folding in the membrane is established. It is clear that most details about the mechanism are yet to be explained by even the more detailed models.

F. Thesis Problems

The overall thrust of my thesis research is to study structure-function relationships in the Band 3 protein using a protein chemical and biophysical approach to ask questions regarding the overall conformation of the protein, and to concentrate on specific functionally important amino acid residues. Mid-way through these studies, in 1985, there was a turning point in the way Band 3 researchers thought about structure-function experiments on this protein, owing to the publication of the sequence of a cDNA of murine Band 3 by Kopito and Lodish (1985a). Especially for residues in the membrane domain, this added a lot to the black boxes that still remained from the partial sequences known by protein sequencing. This thesis represents the work from two major projects, each described in two chapters, and an additional study described in the last chapter.

The long-range goal of the first major project was to develop a procedure for preparing reconstituted Band 3 that would be suitable for functional as well as conformational studies. The approach for obtaining a functionally active reconstitution was one of monitoring functional properties of the protein during the isolation and vesicle formation procedures. A first step would be to isolate the purified protein in a solution of a detergent that is appropriate for the chosen reconstitution procedure. A reconstitution procedure using octyl glucoside dialysis was chosen, since it had been reported to result in large, unilamellar vesicles, and almost complete removal of the detergent (Mimms *et al.*, 1981). It is important to have vesicles with a large internal volume to get good initial rates in flux measurements, though the large size could be a problem in the conformational measurements. With this procedure in mind, Band 3 protein was purified in the nonionic detergent octyl glucoside. Chapter II describes the characterization of the Band 3-octyl glucoside complex. The functionally relevant property of the ability to bind a fluorescent stilbenedisulfonate inhibitor, BADS, was measured. The secondary structure of Band 3 was estimated from circular dichroism measurements, and the oligomeric state of the complex was assessed using several hydrodynamic techniques. This study indicated that although a near normal composition of secondary structural components was maintained, the BADS binding affinity was irreversibly reduced. Also, the oligomeric state was unusually high. The decrease in the BADS binding affinity indicated that the conformation of Band 3 was altered after exposure to high concentrations of octyl glucoside. Since BADS is a transport site inhibitor, it is possible that there would be alterations from native anion transport function as well.

This indicated that a change in the planned reconstitution procedure should be made to avoid this alteration found after exposure of Band 3 to octyl glucoside. At the same time, we became aware of the now published results from Schubert's lab (Scheuring *et al.*, 1986) describing a reconstitution of anion exchange function that displayed essentially native properties for many aspects of the function, overcoming most of the problems from previous reconstitution attempts. Thus, this project evolved into adopting a version of their prescribed reconstitution procedure and carrying out the conformational aspects of the characterization, since they had done a very thorough functional characterization, but no conformational studies on the reconstituted protein.

Our conformational characterization of reconstituted Band 3 is described in Chapter III. To assess the conformation of Band 3 in lipid vesicles we employed two measurements of protein conformation: the far ultraviolet circular dichroism spectrum, which was analyzed to provide the average amounts of defined protein secondary structural forms, and thus indicates the gross conformation of Band 3; and a fluorescence measurement of the binding of the stilbenedisulfonate inhibitor BADS to Band 3, which would detect any changes in the conformation of the stilbenedisulfonate inhibitor binding site. Because the binding site of the competitively inhibiting stilbenedisulfonates is thought to encompass the transport site, this measurement of inhibitor binding is considered a functionally-sensitive conformational property. Our measurements indicated that Band 3-containing vesicles prepared under the prescribed conditions contained protein molecules in which a native inhibitor binding affinity is retained, implying the specific protein conformation within the inhibitor binding site is maintained. Our evidence also indicated that some of the protein in the Band 3-containing vesicles has an apparently altered inhibitor binding affinity. This knowledge helps in the interpretation of the alterations in anion exchange inhibitory potency of stilbenedisulfonates that have been reported by Schubert's group.

In performing the circular dichroism measurements on the Band 3-containing vesicles we encountered the problems that have caused many people to avoid attempting spectral measurements on membrane-bound proteins. The elimination of spectral artifacts caused by the particulate nature of the samples, through instrumental manipulations, data analysis, or changes in the vesicle preparation, is essential to obtaining spectra from which valid secondary structural estimates can be made. We were only able to obtain meaningful spectra when, by collaboration with Dr. Chan Jung, we were able to use a specially designed cell that minimizes the scattering associated with these vesicles. All indications are that the overall composition of secondary structure in the Band 3 in lipid vesicles is not that different from that in detergent solutions. This opens up the possibility of doing further conformational measurements on the reconstituted system, in which the transport studies can be done.

The second major project was an investigation, using the approach of chemical modification, of the role of carboxyl residues in anion exchange. This work was specifically focused on determining the mechanism of inhibition of transport by one carboxyl-selective reagent in particular, the membrane-impermeant carbodiimide ETC. The history and background of the work with this reagent, both within the Reithmeier lab, and in Wieth's lab, has been covered earlier in this chapter. One interesting proposal that resulted from the previous work (Craik and Reithmeier, 1985) was that the two different types of inhibition observed were due to a protein conformational effect (e.g., inward or outward facing transport site) on the consequence of modification of the same residue in Band 3. The problem was, then, to determine what resulted in the differences in inhibitory characteristics in the two cases, and what residue(s) in Band 3 was involved in each type of inhibition. The larger goal would be to study the role of this residue(s) in the various modes of anion transport. The experiments discussed in Chapter IV suggested that the inhibition in the two media (citrate-buffered sucrose, and phosphate-buffered saline) resulted from two very different mechanisms. This caused a major re-evaluation of the existing data, but the modes of inhibition pointed to roles for lysine residues and carboxyl groups in transport.

The experiments in Chapter IV have clearly demonstrated that the majority of the inhibition produced by ETC in citrate-buffered sucrose is due not to protein carboxyl modification, but to carbodiimide-activated citrate modification of a residue in the TrCh -17 fragment. All the evidence is consistent with the residue being lysine a, though we have not yet sequenced the peptide to show this directly. The characteristics of the transport by this modified transporter can potentially clarify the role of the modified residue in anion exchange.

In the absence of citrate, treatment with ETC could also result in inhibition of anion transport. Evidence was that it was due to modification of carboxyl residues, but my work described in Chapter V significantly strengthened the argument, in the finding that nucleophiles afford stabilization of the normally reversible inhibition. An investigation to further define the nature of the inhibition under these conditions, with the goal of identifying the residues involved is also described in this chapter. The usual approaches of using radiolabeled carbodiimide reagent, or radiolabeled nucleophile to obtain labeling were taken. Our attempts at identifying the residues have not been successful. However, we can speculate that the region around this residue(s) is hydrophobic, since only hydrophobic nucleophiles afforded stabilization of inhibition. Our results indicate that the carboxyl group is not within the hydrophobic stilbenedisulfonate binding region since no protection from inhibition by ETC was afforded by DNDS.

Chapter VI presents results of a study that deals with the accessibility of the NEM-unreactive cysteine residue in Band 3, the functional group that has been implicated in allowing the inhibition of osmotic water permeability in red cells by pCMBS. Although this residue is unreactive with NEM, our results indicate that it is relatively accessible in the solubilized protein, since it is reactive with a matrix-bound mercurial sulfhydryl reagent. The results do not provide direct evidence regarding the role of this residue in water transport, but they do put constraints on models of its relative accessibility in the folded structure of the protein. This approach has the potential use for future studies that would address the important question, relating to the folding of Band 3 in the membrane, of the sidedness of this residue.

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II. CHARACTERIZATION OF THE CONFORMATION OF THE BAND 3 PROTEIN IN OCTYL GLUCOSIDE [1]

A. INTRODUCTION

Band 3 protein, the major integral membrane protein of human erythrocytes, is responsible for the exchange of anions such as chloride and bicarbonate across the plasma membrane (Knauf, 1979; Macara and Cantley, 1983). This protein has been purified and characterized in the presence of several detergents (Yu and Steck, 1975a,b; Lukacovic *et al.*, 1981; Nakashima and Makino, 1980). Measurement of the transport activity of purified, detergent-solubilized Band 3, is accomplished by incorporating the protein into phospholipid vesicles. There have been many attempts at reconstitution of Band 3 protein function (Ross and McConnell, 1978; Wolosin, 1980; Lukacovic *et al.*, 1981; van Hoogevest *et al.*, 1983), but many of these reconstituted preparations have achieved only a fraction of the native transport activity, or an activity that has been altered in some other way compared to the native state.

Octyl glucoside has been used successfully for the solubilization, purification and reconstitution of several intrinsic membrane proteins. This detergent has several properties which make it suitable for reconstitution. It is readily dialyzable due to its high critical micellar concentration, (cmc ≅ 20-25 mM [Shinoda *et al.*, 1961]), which allows more efficient removal in reconstitution. This might prevent nonspecific leaks in liposomes due to residual detergent. In addition, it is considered a "mild" detergent that can effectively solubilize membrane proteins without disturbing the native structure of the protein. Because of these properties, octyl glucoside could be expected to be useful as a solubilizing detergent in reconstitution of Band 3, and might improve upon some of the problems encountered in previous attempts. The more recent reconstitutions (Köhne *et al.*, 1983;

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Darmon *et al.*, 1983) have achieved specific transfer rates of up to 80% of those in the native state, as well as activation energies of transport that are close to the native value. This recovery of functionality is more than previous reconstitutions have reported. The reconstitution by Darmon *et al.* (1983) is of particular interest because it used purified Band 3 in octyl glucoside.

It would be useful to know what aspects of the purification and reconstitution procedure could be improved. Alterations in the function of Band 3 protein reconstituted into phospholipid vesicles could potentially be due to either the lack of some specific requirements of the protein, such as some small stabilizing molecule, to the use of inappropriate phospholipids, or to some alteration of the protein during its purification in the presence of detergent. The factors influencing the activity of Band 3 in reconstituted preparations would be better understood if the intermediate state between the native state in the erythrocyte membrane and the reconstituted state, that of the purified, detergent-solubilized protein, were characterized. The necessity for characterizing both the conformation and function of membrane proteins in this intermediate state has been proposed by Tanford and Reynolds (1976). This is especially important since there have been reports of alterations from the native state of some proteins in what are usually regarded as non-denaturing detergents (Gould *et al.*, 1981; Lam and Packer, 1983; Wagner and Gray, 1985; Moriyama and Makino, 1985). The intermediate state has been well characterized for Band 3 in Triton X-100 (Clarke, 1975; Schubert *et al.*, 1983) and in $C_{12}E_8$ and $C_{12}E_9$ (Nakashima and Makino, 1980 [bovine]; Pappert and Schubert, 1983; Reithmeier and Lieberman, 1983) but not in octyl glucoside. Since it is well known that different results may often be obtained when different detergents are used to solubilize the same protein (see, for example, Tanford and Reynolds, 1976; Reynolds, 1982; and Moriyama *et al.*, 1985), a characterization should be done in each particular detergent.

In this study, purified Band 3 in octyl glucoside has been characterized. The detergent binding properties of Band 3 were determined and the oligomeric structure of

Band 3 assessed by sedimentation equilibrium, sedimentation velocity and gel filtration studies. The secondary structure of Band 3 was determined from the far ultraviolet circular dichroism spectrum, and a functionally-sensitive property, that of the integrity of the binding site of a Band 3-specific inhibitor, was assessed by measuring the binding of the stilbene disulfonate inhibitor BADS to Band 3.

B. MATERIALS AND METHODS

1. Materials. $C_{12}E_8$ was purchased from Nikko Chemical Co. (Tokyo), n-octyl-β-D-glucopyranoside from Calbiochem, and n-octyl-β-D-[U-^{14}C]glucopyranoside from New England Nuclear (specific radioactivity: 314 mCi/mmol). Sepharose 4B was obtained from Pharmacia, aminoethyl-Sepharose was prepared according to Shaltiel and Er-El (1973), and pCMB-Sepharose was synthesized as described by Lukacovic *et al.* (1981). Standards used in gel filtration and determination of the Stokes radius were (values from Lemaire *et al.*, 1980): thyroglobulin (8.6 nm), catalase (5.2 nm), and aldolase (4.6 nm) (Pharmacia); apoferritin (6.1 nm) (Schwarz-Mann); *Escherichia coli* β-galactosidase (6.9 nm) and myoglobin (1.9 nm) (Sigma Chemical Co.).

2. Purification of Band 3. All steps were carried out at 4 °C, unless otherwise stated. Erythrocyte membranes were prepared from outdated, packed human red blood cells (kindly provided by the Canadian Red Cross) as described by Dodge *et al.* (1963). The cells were washed at least three times with about 5 volumes of 0.9% NaCl, each time recovering the cells by centrifugation at 5,000 rpm in a Sorvall SS-34 rotor, and removing the residual plasma and buffy coat. The cells were lysed by adding them to 15 volumes of ice-cold 5 mM sodium phosphate, pH 8.0, that contained 0.5 mM phenylmethylsulfonyl-fluoride to minimize proteolysis. The erythrocyte ghost membranes were recovered and rinsed (with the same buffer with no protease inhibitor) using a Millipore Pellicon cassette system (Millipore Corp., Bedford, MA) with an HVLP 0.5 μm filter (Rosenberry *et al.*, 1981). After this point, membranes were recovered by centrifugation at 15,000 rpm in an

SS-34 rotor. Peripheral proteins were extracted by treating the membranes with 20 volumes of 2 mM ethylene diaminotetraacetic acid, pH 12, for 20 minutes. After recovering the membranes, this procedure was usually repeated and the membranes were washed twice with 5 mM sodium phosphate, pH 8.0.

Band 3 was purified by extraction of ghost membranes with 1% $C_{12}E_8$ in 5 mM sodium phosphate, pH 8.0, and chromatography on aminoethyl Sepharose and pCMB-Sepharose in the presence of detergent as described previously (Lieberman and Reithmeier, 1983). One change from this procedure was the addition of a novel method of detergent exchange. The need for detergent exchange is because Band 3 is better solubilized from membranes by $C_{12}E_8$ than by octyl glucoside (R. Reithmeier, personal communication). After rinsing with 0.1% $C_{12}E_8$-containing buffer, detergent exchange of octyl glucoside for $C_{12}E_8$ was accomplished while the protein was bound to either aminoethyl- or pCMB-Sepharose. The column with bound protein was equilibrated with at least three column volumes of buffer containing a known concentration of octyl glucoside from 20 to 40 mM (cmc = 20-25 mM). Following equilibration in the desired concentration of octyl glucoside, the protein was eluted in 50 mM sodium phosphate, pH 8.0, containing the same concentration of octyl glucoside (0.1% 2 -mercaptoethanol was added when eluting from pCMB-Sepharose). The amount of $C_{12}E_8$ remaining associated with the eluted protein following this treatment was below the limits of detection of a chemical assay (<0.015 g $C_{12}E_8$ per g Band 3 protein; Garewal, 1973). Unless otherwise stated, all characterizations were performed with fresh, unfrozen preparations of the protein in 50 mM sodium phosphate, pH 8.0, containing 40 mM octyl glucoside.

3. Detergent Binding. Binding of [^{14}C] octyl glucoside to Band 3 was determined at 4 °C under four conditions: equilibrium dialysis, gel filtration, during elution from an affinity resin, and while bound to an affinity resin. Equilibrium dialysis measurements were carried out in assemblies of 1 ml plexiglass cells (Techni-Lab Mod., Fisher), separated by a Spectra/Por 2 dialysis membrane, that were shaken gently by an arm shaker. A solution of Band 3 (1 mg/ml) in a given concentration of octyl glucoside was dialyzed against the same concentration of detergent containing the radioactive label. Each side was sampled for counting at up to 12 hr intervals. Equilibrium was generally reached within 24 hr for detergent concentrations below the cmc. Above the cmc several days were required to approach equilibrium, and the measurements became increasingly unreliable.

Gel filtration measurements of equilibrium binding (Hummel and Dreyer, 1962) were done by pre-equilibrating and eluting a column (0.9 x 28 cm) of Sepharose CL4B in radioactive octyl glucoside, using the baseline of radioactivity on either side of the protein peak to calculate the specific radioactivity. Samples of each fraction were taken for protein determination and liquid scintillation counting. Protein loading concentrations were 2-3 mg/ml, and the peak concentration of the eluted protein was 0.15-0.2 mg/ml.

Measurements during elution from an affinity resin used Band 3 complexed with pCMB-Sepharose. The column was equilibrated with radioactive octyl glucoside at a given concentration until a stable baseline of radioactivity was reached. The protein was then eluted with the same buffer containing 0.1% 2-mercaptoethanol. Fractions were sampled for protein determination and liquid scintillation counting.

For measurements using protein complexed to an affinity resin, one part protein-saturated pCMB-Sepharose (1:1 slurry in 10 mM octyl glucoside) was added to four parts of a solution containing a given concentration of radioactive octyl glucoside. The amount of bound detergent was determined from the difference between the total and free detergent concentrations. The total octyl glucoside concentration was determined by counting aliquots of the resin suspension. The free concentration of octyl glucoside was determined by counting aliquots of the supernatant after pelleting the resin in a microfuge. 2-mercaptoethanol was added to the suspension (final concentration 0.1%) to release the bound Band 3, and the protein concentration of the supernatant was determined. The value from this determination was corrected for the original volume to obtain the initial protein concentration.

From the value for the maximum amount of detergent bound to the protein (in g/g) an estimate of the fraction of the surface area of a Band 3 monomer covered by detergent was calculated, as described by Clarke (1975). A value of 0.354 nm^2 was used for the cross-sectional area of an octyl glucoside molecule, based on the sugar cross-sectional area calculated from the crystal structure of α-decyl glucoside (Dorset and Rosenbusch, 1981).

4. Analytical Gel Chromatography. Calibration of a Sepharose 4B column (1 cm x 90 cm) with proteins of known Stokes radii (R_s) was carried out at 4 °C in 50 mM sodium phosphate, pH 8.0. Several of the protein standards were run in the same buffer containing either 15 or 40 mM octyl glucoside and no difference in the elution profile was noted. Relative elution volumes (v_e)were determined from continuous monitoring of the absorbance at 280 nm using a Pharmacia UV monitor. The relative elution was expressed as an average partition coeficient, $K_{av} = (v_e - v_o)/(v_t - v_o)$. Blue dextran and 2-mercaptoethanol were used to determine the void (v_o) and total (v_t) volumes, respectively. The Stokes radii for all standards except Mengo virus were taken from LeMaire *et al.*(1980). A Stokes radius for Mengo virus of 14.5 nm was calculated from the intrinsic diffusion coefficient given in Scraba *et al.* (1967).

5. Analytical Ultracentrifugation. Ultracentrifugation was carried out in a Beckman Model E analytical ultracentrifuge. Sedimentation velocity runs (40,000 rpm, 20 °C) used ultraviolet optics at low protein concentrations and Schlieren optics for higher protein concentrations. Sedimentation equilibrium runs (3,000 rpm, 20 °C) used ultraviolet optics. Procedures described by Chervenka (1973) were used for the calculation of weight average molecular weights from sedimentation equilibrium runs, using a $\bar{\upsilon}$ for the protein-detergent complex calculated as described below.

The molecular weight of the protein-detergent complex was calculated from the sedimentation coefficient, $s_{20,w}$, Stokes radius, R_s, and partial specific volume, $\bar{\upsilon}$, using the equation (Tanford *et al.*, 1974):

$$M_c = \frac{6\pi\eta N_A s^{o}_{20,w,c} R_{s,c}}{1 - \bar{\upsilon}_c \rho} \qquad \cdots\cdot [\text{II-1}]$$

where the subscript c denotes values for the protein-detergent complex, η is solvent viscosity, N_A is Avogadro's number, and ρ is solvent density. The partial specific volume of the protein-detergent complex ($\bar{\upsilon}_c$) was calculated (assuming that the partial specific volumes of the protein and detergent components are additive), using the equation (Tanford *et al.*, 1974):

$$\bar{\upsilon}_c = \frac{\bar{\upsilon}_p + \delta_d \bar{\upsilon}_d}{1 + \delta_d} \qquad \cdots\cdot [\text{II-2}]$$

where $\bar{\upsilon}_p$ and $\bar{\upsilon}_d$ are the partial specific volumes of the protein and detergent, and δ_d is the amount of bound detergent (g/g protein). A value for $\bar{\upsilon}_p$ of 0.74 cm^3/g, from measurements of acetic acid-solubilized Band 3 (Dorst and Schubert, 1979) was used. From density measurements of solutions of octyl glucoside at six concentrations above and below the cmc, the $\bar{\upsilon}_d$ was calculated to be 0.85 cm^3/g, in close agreement with a published value (Watts *et al.*, 1982). The molecular weight of the protein component of the complex was obtained after correction for the bound detergent using $M = M_c/(1+\delta_d)$ (Tanford *et al.*, 1974).

6. Circular Dichroism. Circular dichroism spectra were recorded on a Jasco J-500C spectropolarimeter with a Jasco DP-500N data processor. Spectra were determined from a minimum of two scans at 25 °C using a 0.05 cm pathlength cell. The α-helical and β-sheet contents were estimated from equation [II-3] using the experimental mean residue ellipticity values, $[\theta]$, at wavelengths of 215, 220 and 225 nm, and using the reference protein values provided by Chen *et al.* (1972).

$$[\theta]_{\lambda} = f_{H}[\theta]_{H\lambda} + f_{\beta}[\theta]_{\beta\lambda} + f_{R}[\theta]_{R\lambda} \qquad \cdots\text{[II-3]}$$

The fs are the fractions of helix (H), β form and random coil (R) at each wavelength. A mean residue molecular weight of 113 (Yu and Steck, 1975a), which was based on amino acid composition, was used in the calculation.

7. BADS Binding. BADS binding to Band 3 in solution was determined by fluorescence energy transfer, as previously described (Rao *et al.*, 1979; Lieberman and Reithmeier, 1983). All measurements were made at 20 °C using a Perkin-Elmer MPF-44B fluorescence spectrophotometer, and were corrected for dilution, background fluorescence and fluorescence quenching. Protein samples were diluted with 28.5 mM sodium citrate, pH 7.4, for the assay. Excitation of the protein was done at 280 nm and the fluorescence was measured at 450 nm. Excitation of BADS directly at 340 nm was not used because the background fluorescence was higher and increased dramatically above the detergent's cmc, which made the background correction more difficult. Presumably this was due to BADS partitioning into micelles.

8. Analytical Procedures. Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (1970). 10% polyacrylamide slab gels were run at constant current (20 mAmp for 0.75 mm thickness). The gels were stained with a solution of 0.1% Coomassie Brilliant Blue-R in 25% isopropanol, 10% acetic acid and destained in 25% methanol, 10% acetic acid. Densitometry of the stained gel was performed at 600 nm using a Gilford 250 spectrophotometer with a linear scanning attachment. Protein concentrations were determined by the method of Lowry *et al.* (1951) in the presence of 1% sodium dodecyl sulfate, and using bovine serum albumin fraction V (Sigma Chemical Co.) as a standard. Scintillation counting was performed in a Beckman LS7800 scintillation counter with an external standard method of quench correction. Solution densities were measured at 20.0 ± 0.01 °C on an Anton Paar precision density meter, model DMA 60 (Anton Paar, Graz, Austria). Viscosity measurements on solutions were carried out in a 10 mL capacity Oswald viscometer held at 20 °C in a constant temperature water bath.

C. RESULTS AND DISCUSSION

1. Octyl Glucoside Binding

The preparation of Band 3 in octyl glucoside used in these experiments was over 95% pure, as judged by sodium dodecyl sulfate gel electrophoresis (Figure II.1). The binding of octyl glucoside to this preparation of Band 3 was measured over a range of free detergent concentrations (Figure II.2), using the methods of equilibrium dialysis, gel filtration, during elution from an affinity resin, and while complexed to an affinity resin (described in Materials and Methods). Below the cmc of the detergent all four methods of measurement gave approximately the same detergent binding curve, clearly indicating that Band 3 bound monomers of octyl glucoside. Above the cmc, the equilibrium dialysis results were variable and did not show saturation of binding. In contrast, the results by gel filtration and both versions of affinity chromatography showed saturation of binding, with a maximum of 0.64 g octyl glucoside bound per g Band 3 protein. The saturable level of binding corresponds to about 208 moles octyl glucoside bound per mole Band 3, well above the aggregation number of octyl glucoside of 52 that has been reported (Roxby and Mills, 1981). The results were similar whether the protein was free in solution or bound to pCMB-Sepharose, indicating that interaction of Band 3 with this resin does not alter its detergent binding properties. The fact that binding occurs below the cmc indicates that monomers of octyl glucoside interact with discrete binding sites on Band 3, and that Band 3 does not simply insert into a detergent micelle (Tanford and Reynolds, 1974). The extent of binding indicates a large hydrophobic surface area on the protein, which was to be expected for an intrinsic membrane protein. An estimate of the fraction of the surface area of a Band 3 monomer covered by detergent is calculated to be 0.64. Our results in octyl glucoside are similar to those of Clarke (1975), who reported that Band 3 binds Triton X-100 below the cmc, and calculated the fraction of the surface area of the protein interacting

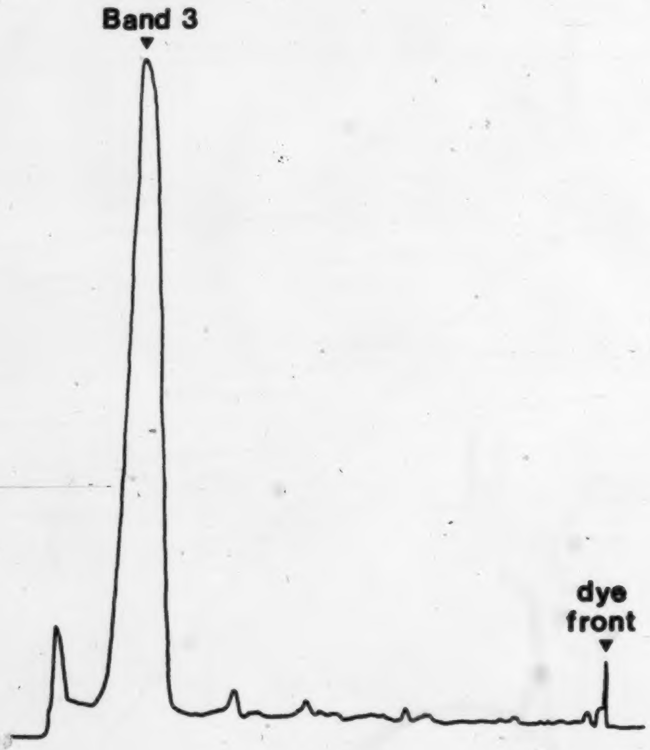

Figure II.1. Sodium dodecyl sulfate gel electrophoresis of Band 3 protein (50 µg) isolated in octyl glucoside. The gel contained 10% polyacrylamide, was stained with Coomassie brilliant blue and was scanned at 600 nm.

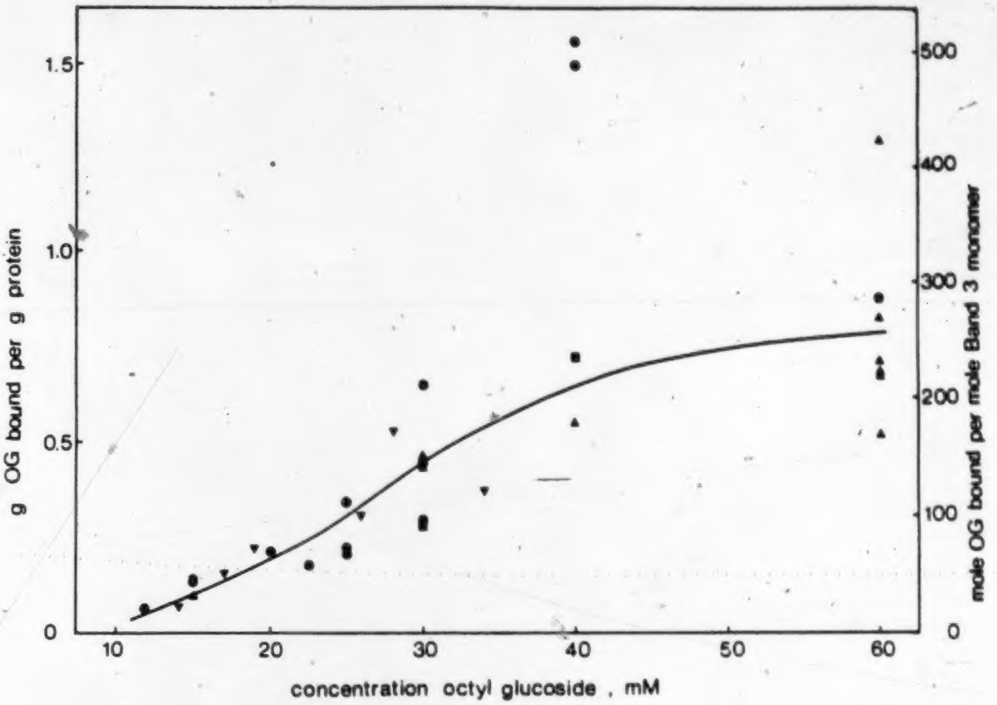

Figure II.2. Binding of octyl glucoside to Band 3 as a function of octyl glucoside concentration. Detergent binding was measured at 4°C in 50 mM sodium phosphate, pH 8.0, with the stated concentration of octyl glucoside, by equilibrium dialysis (●), gel filtration (■), during elution from pCMB-Sepharose (▲), or while bound to pCMB-Sepharose (▼). Error bars, for the equilibrium dialysis measurements only, represent the standard deviation for three or more determinations.

with the detergent (at maximal binding) to be 0.50 to 0.65. The amount of bound detergent must be considered in the hydrodynamic analysis of the Band 3-octyl glucoside complex.

2. Hydrodynamics

It is important to know the oligomeric state of the protein in detergent solution, especially if any investigation of the relationship between oligomeric state and function is to be made, either in detergent solution or in a reconstituted state. Sedimentation velocity runs of Band 3 in octyl glucoside produced a single broad boundary. In 40 mM octyl glucoside, where the maximum amount of detergent is bound, the sedimentation coefficient decreased with increasing protein concentration (Figure II.3), giving an intrinsic sedimentation coefficient ($s°_{20,w}$) of 32 S. This suggests that Band 3 isolated in octyl glucoside is in a very high oligomeric state. Sedimentation coefficients reported for Band 3 in other non-ionic detergents are much lower: in Triton X-100 an $s_{20,w}$ of 6.9 S has been reported and ascribed to a stable noncovalent dimer (Clarke, 1975); in $C_{12}E_8$ two components were resolved with $s_{20,w}$ of 7.5 S and 11.1 S, corresponding to dimer and tetramer (bovine Band 3; Nakashima and Makino, 1980). Pappert and Schubert (1983) obtained results similar to those of Nakashima and Makino in $C_{12}E_8$, but observed a single, broad boundary rather than two components, with the $s_{20,w}$ increasing from 3.8 S to 8 S as the protein concentration was increased. They obtained similar results in Ammonyx-LO (Pappert and Schubert, 1983). It is evident that Band 3 in octyl glucoside is in an oligomeric state much higher than tetramer, which is the highest oligomeric structure

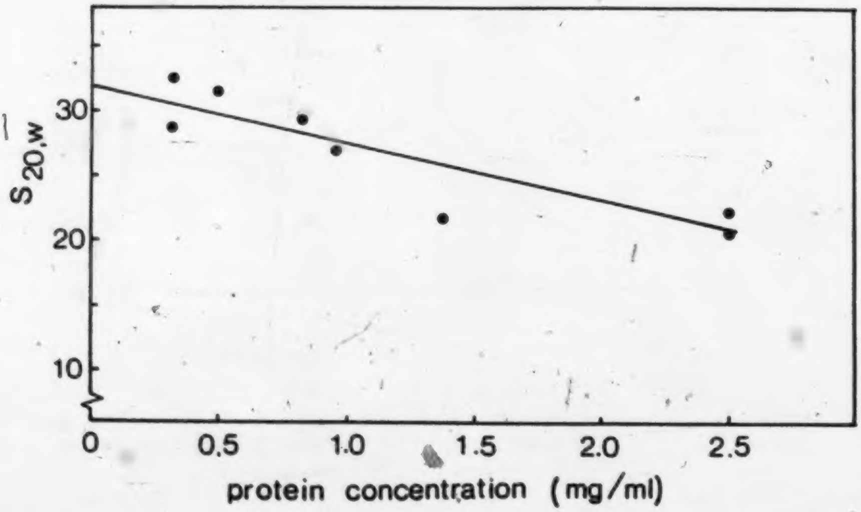

Figure II.3. Dependence of $s_{20,w}$ on protein concentration for Band 3 purified in 40 mM octyl glucoside. Sedimentation was carried out at 20 °C at 40,000 rpm, in 50 mM sodium phosphate, pH 8.0, 40 mM octyl glucoside.

usually observed by sedimentation in other nonionic detergents. Another major difference between the sedimentation behaviour of Band 3 in octyl glucoside and in $C_{12}E_8$ is that no decrease in $s_{20,w}$ was noted at lower protein concentrations in octyl glucoside, in contrast to observations by Pappert and Schubert (1983). It must be noted that we studied sedimentation only down to a protein concentration of 0.2 mg/ml, while they went down to 0.05 mg/ml. It is possible that dissociation might occur below the lowest protein concentration used in our study. However, it is the sedimentation coefficient at protein concentrations ≥ 0.2 mg/ml that would be of interest for use in estimates of the molecular weight since the Stokes radius has been determined at these concentrations.

The Band 3-octyl glucoside complex, at maximum detergent binding (40 mM octyl glucoside), eluted from a Sepharose 4B column as a broad peak of $K_{av} \cong 0.40$, with the values of K_{av} at half-height ranging from 0.24 to 0.56 in one instance. The elution profile sometimes contained a component (up to 10% of the total absorbance at 280 nm) that eluted at the void volume. From a calibration curve (Figure II.4) using water-soluble proteins of known Stokes radii, the elution position of the major component corresponded (as estimated by extrapolation) to a Stokes radius of 10.1 nm. This is about 1.3 times the Stokes radius determined for the protein in Triton X-100 (Reithmeier, 1979).

The results from sedimentation velocity and gel filtration measured in 40 mM octyl glucoside were used to calculate a molecular weight of the Band 3-octyl glucoside complex of 1.81×10^6. The molecular weight of the complex was corrected for the mass contributed by the bound detergent to obtain a molecular weight of the protein component (described in Materials and Methods). The maximum value of 0.55 g octyl glucoside bound per g Band 3 protein was used to correct the molecular weight of the complex, since very close to the maximum amount of detergent is bound to the protein at 40 mM octyl glucoside. This gave a molecular weight for the protein component of the Band 3-octyl glucoside complex of 1.17×10^6. Based on a monomeric molecular weight of 95,000 the calculated molecular weight corresponds to a dodecamer.

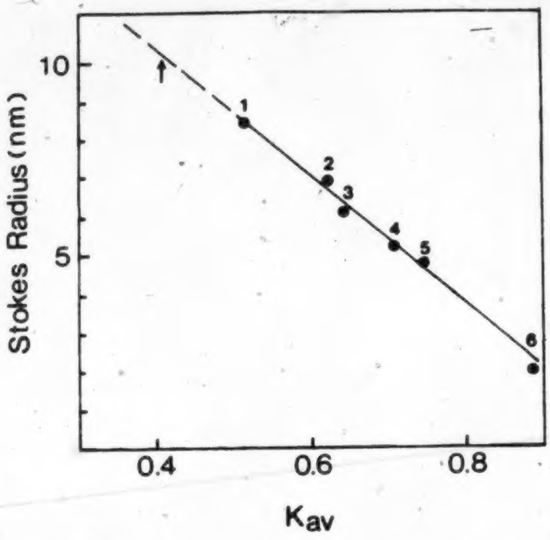

Figure II.4. Determination of the Stokes radius of Band 3 in octyl glucoside. A column of Sepharose 4B (1 x 90 cm) was calibrated in 50 mM sodium phosphate, pH 8.0, at 4 °C Each point represents 1 to 4 determinations in this buffer. Addition of 40 mM octyl glucoside to the buffer did not change the elution positions of the standards. The calibration is presented as a plot of Stokes radius, R_s *vs.* K_{av}. The relative elution position of Band 3-octyl glucoside complex in the above buffer with 40 mM octyl glucoside, an average of four determinations, is denoted by the arrow. Standard proteins: 1, thyroglobulin; 2, β-galactosidase; 3, apoferritin; 4, catalase; 5, aldolase; 6, myoglobin

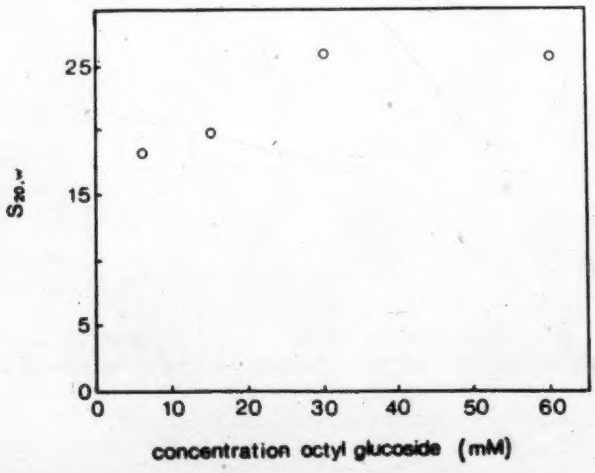

Figure II.5. Dependence of sedimentation coefficient of Band 3 on octyl glucoside at protein concentrations of 0.71-0.75 mg/ml. Sedimentation conditions were as described in Figure II.3.

The sedimentation coefficient of the Band 3-octyl glucoside complex increased with increasing octyl glucoside concentration from 6 to 60 mM (Figure II.5). However, an analysis similar to that above using $s^\circ_{20,w}$ and R_s determined in 15 mM octyl glucoside ($s^\circ_{20,w} = 18.2$ S, $R_s = 9.7$ nm, aggregation number ≅ 10) indicated that this could largely be accounted for by the increase in mass that would accompany detergent binding. This suggests that there is no drastic change in oligomeric state over the range of octyl glucoside concentrations used here (6 mM to 60 mM). Certainly, the higher octyl glucoside concentrations did not dissociate Band 3 to lower oligomers.

The broad boundary observed in sedimentation velocity and gel filtration suggests that the oligomeric state may be heterogeneous. This was confirmed by sedimentation equilibrium. A curvilinear plot of ln c versus r^2 was produced (Figure II.6), indicating a

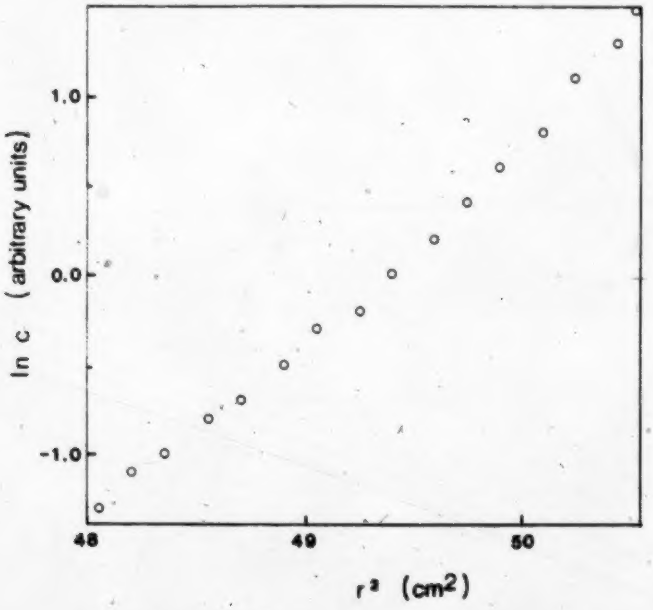

Figure II.6. Sedimentation equilibrium centrifugation of Band 3 in 40 mM octyl glucoside. The initial protein concentration was 1.37 mg/ml, and sedimentation was performed at room temperature with a rotor speed of 3,000 rpm, in 50 mM sodium phosphate, pH 8.0, 40 mM octyl glucoside. The ln of the protein concentration (c) is plotted as a function of the square of the distance from the center of rotation (r^2).

molecular weight range from 1.8 x 10^6 to 3.15 x 10^6 for the Band 3-octyl glucoside complex. A range of (1.16 - 2.0) x 10^6, with an average of 1.6 x 10^6, was calculated for the protein component. Based on a monomeric molecular weight of 95,000 this corresponds to a range from a 12-mer up to a 22-mer with an average of a 16-mer. These results are in reasonable agreement with the molecular weight calculated from combined $s^{\circ}_{20,w}$ and R_s, although the sedimentation equilibrium results indicate a somewhat higher oligomeric state. This higher oligomeric state may have been created by the overnight exposure of the Band 3 to room temperature that occurred during the sedimentation equilibrium experiments.

The results of the hydrodynamic analysis indicate that the oligomeric state of Band 3 isolated in octyl glucoside is heterogeneous and very high, having an average aggregation number of n = 12-16. This is much higher than any other oligomeric state reported for solubilized Band 3. Pappert and Schubert (1983) report that their sedimentation equilibrium data of Band 3 in $C_{12}E_8$ fits quite well to a model of a monomer/dimer/tetramer self-association equilibrium. Although they point out that their data do not rule out that higher oligomers (hexamers, octamers) may take part in the equilibrium, their sedimentation coefficients at similar protein concentrations are much lower than ours in octyl glucoside. Earlier work on acetic acid-solubilized Band 3 (Dorst and Schubert, 1979) reported $s_{20,w}$ values of up to 20 S, which they ascribed to Band 3 octamers, and indicated that, under appropriate conditions, hexamers and octamers contributed to the observed monomer/dimer/tetramer equilibrium.

It is generally agreed that Band 3 exists as a mixture of dimers and tetramers in the erythrocyte membrane under physiological conditions (Jennings, 1984). Crosslinking studies in intact cells produced dimers, but no higher oligomers (Staros and Kakkad, 1983). Freeze-fractured membranes show particles of tetrameric size (Weinstein *et al.*, 1980), and the resonance energy transfer data on the distance between stilbene disulfonate sites suggests that dimers and tetramers of Band 3 exist in the membrane (Macara and Cantley, 1981). Our results suggest that Band 3 in octyl glucoside is in a much higher oligomeric state than in the erythrocyte membrane. This indicates that octyl glucoside affects the oligomeric state of proteins in different ways. It has been found to dissociate some proteins, for example, pilin arrays (Watts *et al.*, 1982), and cytochrome P-450-NADPH-cytochrome P-450 reductase complexes (Dean and Gray, 1982). The high oligomeric state of Band 3 in octyl glucoside might prevent the use of this detergent for reconstitution of single functional units of Band 3. However, it is possible that reassociation of the protein with phospholipids could allow dissociation, since phospholipid has been found to dissociate oligomers of the sarcoplasmic reticulum Ca^{2+}-ATPase (Andersen *et al.*, 1983).

3. Circular Dichroism

The far ultraviolet circular dichroism spectrum of Band 3 in octyl glucoside, shown in Figure II.7, is typical of a protein containing a high amount of α-helix, as indicated by the double trough between 200 and 250 nm. Calculation of the amount of secondary structure (equation [II-3], Materials and Methods) indicated that the protein has an α-helical content of 40% in 2 mM octyl glucoside. With increasing concentrations of detergent, the shape of the spectrum remained the same and there was a slight increase in negative ellipticity. In 60 mM octyl glucoside (maximum detergent bound), the molar ellipticities at the minima of 208 and 217.5 were approximately 5% and 7% more negative than in 2 mM octyl glucoside, indicating an α-helical content of 45%. An α-helical content of 40 to 45% is similar to that reported for Band 3 purified in other detergents: 41% in $C_{12}E_8$ (Oikawa *et al.*, 1985), and 43% in Ammonyx-LO (Yu and Steck, 1975a). It has been claimed that the native structure of the protein was retained in this latter detergent (Yu and Steck, 1975a).

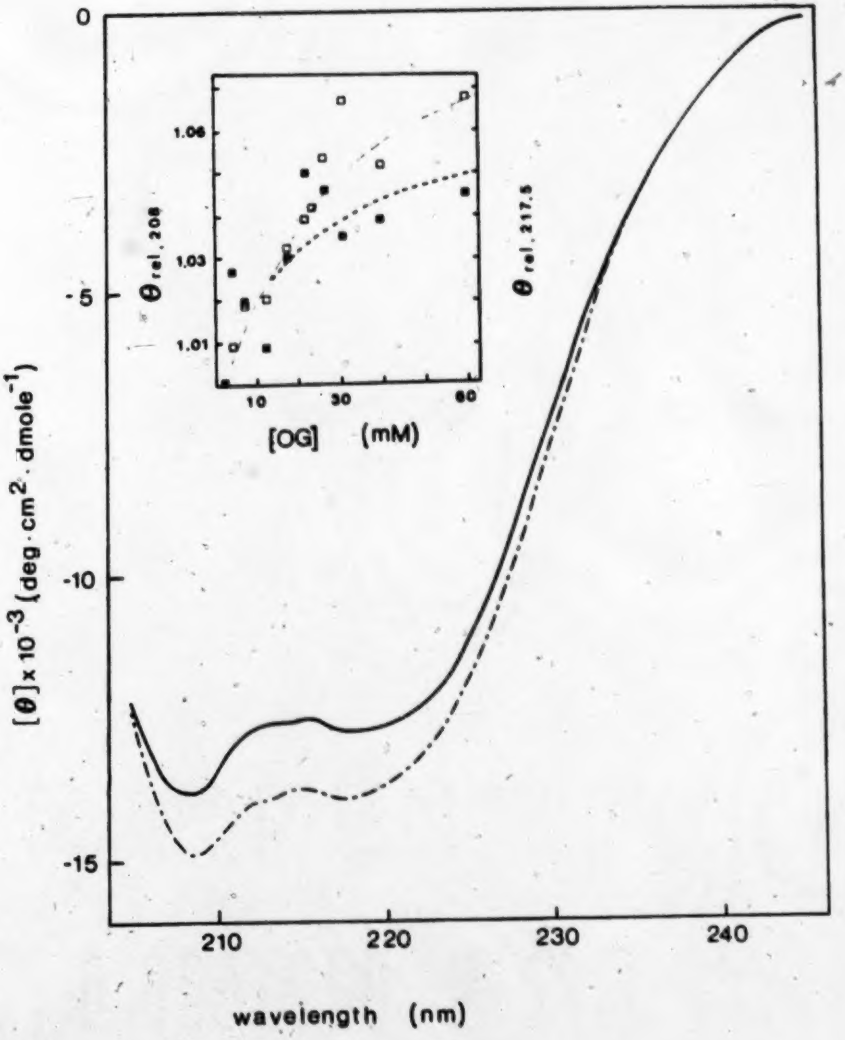

Figure II.7. Far ultraviolet circular dichroism spectrum of Band 3 in octyl glucoside concentrations of 2 mM (—) and 60 mM (- -). Spectra were recorded at 25 °C in 50 mM sodium phosphate, pH 8.0, using a cell of pathlength 0.05 cm. The results are the average for two samples of protein concentrations 0.562 and 0.525 mg/ml. Inset: effect of octyl glucoside concentration on the relative ellipticity at 208 nm (-- ■ --) and at 217.5 nm (- □ -).

Our results therefore suggest that there are no gross alterations in the secondary structure of Band 3 when it is purified in octyl glucoside. The slight increase in negative ellipticity observed at higher concentrations could indicate an actual increase in the amount of secondary structure.

4. BADS Binding

The anion transport activity of Band 3 can be measured only when the protein is inserted in a sealed membrane. However, the functional integrity of the soluble purified protein in octyl glucoside was determined by the ability of Band 3 to bind BADS, a potent inhibitor ($K_i = 1\ \mu M$) of anion transport. This provides an estimate of the integrity of the stilbene disulfonate binding site that can be compared to the value for the native protein. The measurement of BADS binding in solution is based on a fluorescence enhancement assay in which excitation of tryptophan residues in the protein at 280 nm results in energy transfer to BADS that is bound to the hydrophobic inhibitor binding site of Band 3. The BADS fluorescence emission was measured at 450 nm.

Band 3 purified in octyl glucoside bound BADS, as shown by the saturable fluorescence enhancement upon addition of increasing concentrations of BADS (Figure II.8). The affinity of BADS binding obtained from such a titration varied with the concentration of octyl glucoside present in the assay. Figure II.9 shows the effect of increasing concentrations of octyl glucoside on the affinity of BADS binding for preparations purified in three different concentrations of octyl glucoside. Preparations that were purified in 2 mM octyl glucoside, and exposed to a maximum concentration of 20 mM octyl glucoside during detergent exchange, bound BADS with high affinity (0.8-1.5 μM). This compares quite well to the affinity of BADS for Band 3 in membranes ($K_d \cong 1\ \mu M$) and the inhibition constant of BADS inhibition of phosphate uptake in cells ($K_i \cong 1\ \mu M$) (Lieberman and Reithmeier, 1983). It is also consistent with reported results in which direct solubilization of ghosts with octyl glucoside (0.1% or ≅ 3.5 mM) had no effect on

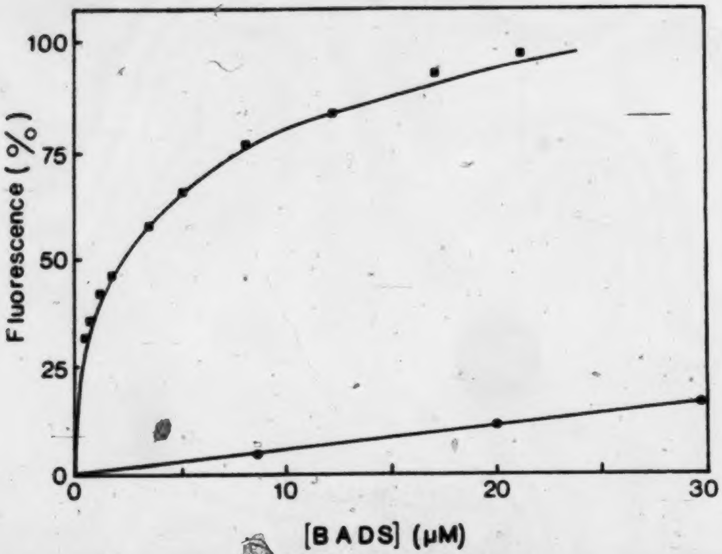

Figure II.8. Binding of BADS to purified Band 3 as determined by fluorescence energy transfer. Band 3 (■) purified in 20 mM octyl glucoside was diluted with 28.5 mM sodium citrate, pH 7.4, for the assay (final concentration 0.7 mM octyl glucoside). Background fluorescence (●) of BADS in 20 mM octyl glucoside. Excitation was at 280 nm and emission was measured at 450 nm.

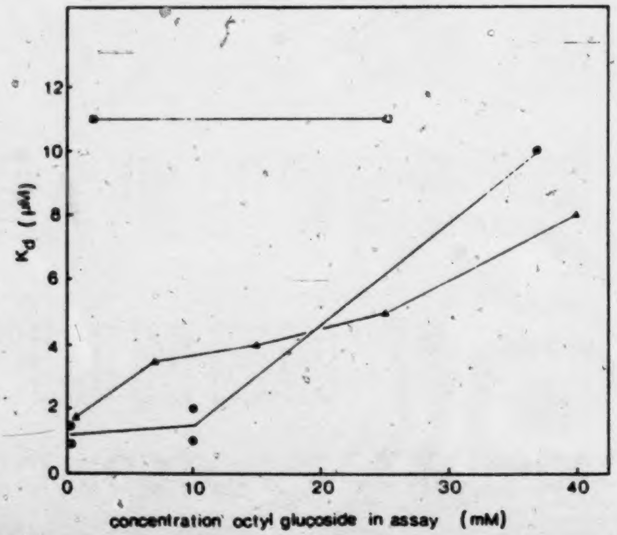

Figure II.9. Effect of concentration of octyl glucoside on the affinity of BADS binding to Band 3. The K_d of BADS binding was determined using the fluorescence enhancement assay at the stated assay concentration of octyl glucoside for Band 3 prepared in 2 mM (●), 15 mM (▲) and 40 mM (■) octyl glucoside. The protein prepared in 40 mM octyl glucoside was exposed to 250 mM octyl glucoside for 6 h at 4 °C prior to assay at 25 mM octyl glucoside (□).

the affinity of BADS binding (Lieberman and Reithmeier, 1983). Increasing the octyl glucoside concentration gradually increased the K_d of BADS binding to 8-10 μM at an octyl glucoside concentration of approximately 40 mM (Figure II.9, preparations in 2 mM and 15 mM). Above this detergent concentration it became technically difficult to do the measurements, due to the high background fluorescence of BADS.

The increase in K_d observed in higher octyl glucoside concentrations did not appear to be reversible. In a preparation purified in an octyl glucoside concentration above the cmc (40 mM), then diluted to a concentration below the cmc for the binding assay, the K_d remained at ≅11 μM, rather than decreasing to the K_d measured at the same assay concentration for preparations in 2 mM or 15 mM octyl glucoside. Exposure to an even higher concentration of octyl glucoside (to 250 mM) for 6 h, conditions which might approximate those used in reconstitution from octyl glucoside, did not have any further effect on the affinity of BADS binding. It should be noted that the maximum fluorescence enhancement was the same for the measurements done at assay concentrations of octyl glucoside of 25 mM or lower, indicating that the number of BADS binding sites did not change as the affinity changed. However, exact numbers of binding sites per protein cannot be determined using this method of measurement. The decrease in affinity may be due to some alteration in the conformation of the inhibitor binding site of the protein, or to a more direct interaction of octyl glucoside with the inhibitor binding site. The former seems more likely, since the altered affinity is not reversible upon dilution into low concentrations of detergent. Moriyama and Makino (1985) have concurrently reported a measurement of BADS binding to bovine Band 3 in octyl glucoside (as well as several other detergents). Their results indicate that the K_d of BADS (measured by a method almost exactly like our method) was increased almost 30-fold in octyl glucoside (0.5% OG in the assay, K_d = 28.8 μM). Other nonionic or zwitterionic detergents produce even higher K_d s, or abolished binding, and the K_d is 12.7 μM (10-fold increased)when Band 3 is in $C_{12}E_9$. Thus, their results of a reduction in the affinity of BADS binding in octyl glucoside agree with ours.

There have been previous reports of alterations in stilbene disulfonate binding and inhibitory potency in reconstituted Band 3 preparations. It was found that the concentrations of DIDS or DNDS required to inhibit transport in Band 3 proteoliposomes (100 μM DIDS or 500 μM DNDS) were an order of magnitude higher than those required in cells (Cabantchik *et al.*, 1980; Wolosin, 1980; van Hoogevest, 1983). Köhne *et al.* (1981) found that their reconstituted preparation had a lowered and more heterogeneous affinity for DNDS, with an I_{50} value estimated to be about sixfold higher than that found in cells. Although none of these reconstitution procedures involved exposure to octyl glucoside, it is intriguing that they displayed a similar reduction in stilbenedisulfonate binding (or inhibitory) capacity that is similar to what we observe for Band 3 in octyl glucoside. This may be due to a structural alteration in the protein by the detergent.

D. CONCLUSIONS

Although Band 3 purified in octyl glucoside retains a significant amount of α-helical content, the affinity of BADS for the stilbenedisulfonate binding site is reduced tenfold. In fact, a 5% increase in negative ellipticity at 208 and 217.5 nm was observed at high concentrations of detergent, indicative of an increase in α-helical content. This change in secondary structure is consistent with the idea that a structural alteration led to the observed decrease in affinity for BADS. It must be noted that addition of 1% sodium dodecyl sulfate to purified Band 3 has been reported to have had little effect on the circular dichroism spectrum (Oikawa *et al.* 1985), yet it destroyed the stilbenedisulfonate binding site (Lieberman and Reithmeier, 1983). In that case, and the one presented here, the measurement of inhibitor binding affinity was more sensitive to the alterations than was the spectroscopic structural indicator of an estimate of α-helical content from the circular dichroism spectrum. While circular dichroism and estimation of secondary structure is an important part of the characterization of a detergent-solubilized membrane protein, the net circular dichroism spectrum may not be sensitive to small structural changes that could result in functional alteration. Along the same idea, it has been noticed that limited changes (i.e., production of a nonnative, but still significantly ordered structure) are sometimes reversible (Weber and Kuter, 1971; Boodhoo and Reithmeier, 1984; Fröhlich and Jones, 1987). Nevertheless, the alteration described here is not reversible upon reduction of the octyl glucoside concentration.

There are a growing number of examples of membrane proteins for which an alteration from native function or structure (or inhibitor binding) is produced in some nonionic detergents. A few examples are bacteriorhodopsin, for which alterations in the absorption spectrum in several nonionic detergents, including β-octylglucoside, have been observed (Lam and Packer, 1983), and cytochrome P-450_{LM2} in octyl glucoside, for which both spectroscopic and activity changes were noted (Wagner and Gray, 1985). A situation similar to that for the cytochrome P-450 is also observed for the erythrocyte insulin receptor in octyl glucoside (Gould *et al.*, 1981).

Thus, this emphasizes that an assay of protein function (or a functionally-sensitive property) is necessary to evaluate whether purification in the presence of a particular detergent results in alteration of the native properties. This point has been made by Tanford and Reynolds (1976), who stressed the importance of carrying out a structural and functional characterization of the intermediate state for a purified, detergent-solubilized membrane protein that is to be reconstituted. Such a characterization would make it possible to determine whether alterations in structure or function that might be observed in the reconstituted state had occurred before or after this intermediate state. It would then be possible to decide which aspect of the purification and reconstitution needed improvement.

Reconstitution studies which use Band 3 in octyl glucoside (Darmon *et al.*, 1983; Darmon *et al.*, 1985) must take into account the oligomeric state and the alteration in inhibitor binding that we detect in the octyl glucoside-solubilized protein . This also suggests that any future reconstitution studies on Band 3 protein might want to avoid the use of octyl glucoside above its cmc, or at least to investigate the effects of this detergent in the presence of other detergents or phospholipids. An example that would have bearing on this issue is the reconstitution reported by Schubert's lab (published after the findings described in this chapter were published), which uses a mixture of Triton X-100 and octyl glucoside (Scheuring *et al.*, 1986; Scheuring *et al.*, 1987). The work in the following chapter will address this issue.

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III. CONFORMATIONAL STUDIES ON BAND 3 RECONSTITUTED INTO LIPID VESICLES

A. INTRODUCTION

Having a method that provides conformational information is essential to structure-function studies of membrane proteins. The methods used for membrane proteins have been chiefly absorption spectroscopy and circular dichroism, but some studies have employed fluorescence, infrared or raman spectroscopy, nuclear magnetic resonance spectroscopy, or x-ray diffraction. Most of the conformational studies on Band 3 protein have used the technique of circular dichroism, which is one of the most sensitive measures of the solution conformation of proteins. All the CD studies on purified Band 3 protein have been done on the solubilized protein in detergent solution. Near ultraviolet CD and far ultraviolet CD studies have been done on bovine Band 3 in $C_{12}E_9$ (Nakashima and Makino, 1980a,b), and far ultraviolet CD has been done on human Band 3 in dimethyldodecylamineoxide (Yu and Steck, 1975), in $C_{12}E_8$ (Lieberman and Reithmeier, 1983; Oikawa, Lieberman and Reithmeier, 1985) and in octyl glucoside (Werner and Reithmeier, 1985). In all cases, analysis of the far ultraviolet CD indicates isolated Band 3 contains about 45% α-helix.

The fluorescence studies of stilbenedisulfonate inhibitor binding to Band 3 also have provided important information about the nature of the stilbenedisulfonate inhibitor binding region, which provides a convenient assay for the native structure of Band 3. Because the inhibitor binding site is thought to encompass the "transport site" of Band 3 (Passow, 1986), these measurements are expected to be uniquely sensitive to conformational alterations near the transport site. Stilbenedisulfonate binding affinities to Band 3 in the erythrocyte membrane and in detergent solutions have been measured (Rao *et al.*, 1979; Lieberman and Reithmeier, 1983). Alterations in the affinities have been detected in the presence of some detergents (Lieberman and Reithmeier, 1983; Werner and Reithmeier, 1985; Moriyama and Makino, 1985).

Conformational studies on purified Band 3 have been done only in detergent solutions, and never in a native millieu. Detergents are rarely a close substitute for the native lipids, and even detergents generally regarded as nondenaturing can in some cases alter or fully disrupt the functional properties of membrane proteins (Werner and Reithmeier, 1985; Mao and Wallace, 1984 ; others are discussed in chapter II). It is clearly important to extend structural studies done on purified Band 3 in detergents to Band 3 that is imbedded in a membrane, which would be more representative of the native environment of the protein.

The lack of structural studies on Band 3 in a membrane-bound state is suspected to be due not to a lack of interest but, more likely, to the difficulties in applying spectroscopic techniques to, and the hazards in interpretation of the results in, samples of membrane suspensions. A major difficulty in obtaining meaningful CD spectra of particulate samples, such as membrane vesicles, is that the presence of the vesicles creates distortions in the spectrum of the protein. The major spectral artifacts arise from the large size of the membrane particles relative to the wavelength of the light, resulting in differential light scattering, and from the inhomogeneous distribution of the proteins in the membranes, resulting in differential absorption flattening. Theoretical and practical aspects of the analysis of data containing these spectral artifacts have been studied and reviewed (Gordon and Holzwarth, 1971; Long and Urry, 1981, and references within; Schneider, 1973, and references within). Techniques for instrumentally eliminating the differential scattering effects have been developed (Bustamante *et al.*, 1983; Reich *et al.*, 1980), and advances in the area of minimizing, and correcting for, the absorption flattening artifacts have been made by Wallace and co-workers (Wallace and Mao, 1984; Mao and Wallace, 1984; Wallace and Teeters, 1987). The latter authors' work indicates that the optical artifacts are minimal when the protein (bacteriorhodopsin) is present in small unilamellar vesicles.

Using reconstituted Band 3, that is, a preparation of purified Band 3 inserted into a lipid bilayer, to do conformational studies provides several advantages. An obvious advantage discussed above is that the purified protein is in a native-like environment. Reconstituted vesicles also provide a form in which native function can be assessed, or in which functional studies may have been done. The conformational studies would complement functional studies done on the same preparation. Several attempts at reconstitution of the anion exchange function of Band 3 have been reported (Ross and McConnell, 1978; Wolosin, 1980; Cabantchik *et al.*, 1980; Lukacovic *et al.*, 1981; Köhne *et al.*, 1981, 1983; van Hoogevest *et al.*, 1983; Darmon *et al.*, 1983; Darmon *et al.*, 1985). Most of these have resulted in properties of anion exchange that are altered in some way from those measured in erythrocytes. In addition to failing to produce some of the basic characteristics of native anion transport, all reports of the sensitivity of the transport to specific inhibitors noted an increase in the inhibitor concentration required for half-maximal inhibition (as alluded to in the discussion of chapter II). A recent report (Scheuring *et al.*, 1986; and modifications, Scheuring *et al.*, 1987, 1988) has demonstrated a reconstitution in which the essential features of anion exchange closely resemble those in erythrocytes.

Scheuring *et al.* (1986) have done a thorough characterization of the transport function, but have not examined the structure of the Band 3 in their functionally reconstituted preparations. It is partly for this reason that a conformational characterization on Band 3-containing vesicles prepared by their procedure was carried out. There was an additional impetus for doing part of the experiments we describe. Scheuring's reconstitution method makes use of a mixture of detergents: OG, a detergent having a high cmc, and Triton X-100, an alkylphenyl polyoxyethylene detergent having a low cmc. We had determined that Band 3 in OG alone is highly aggregated and has an irreversibly reduced affinity of inhibitor binding. Thus, it was important to confirm that inhibitor binding is not impaired during the reconstitution procedure using a mixture of detergents.

The main question that is addressed by the work described in this chapter is: has the conformation of Band 3 been retained in this reconstituted phospholipid vesicle preparation? This is divided into two, more specific, questions:

1. What is the effect of reconstitution of Band 3 protein by this method on the binding of the stilbenedisulfonate inhibitor, BADS? This question has a more specific component to it, which is to determine whether the alteration in BADS binding that we detected in OG is prevented by this reconstitution procedure.

2. What is the effect of re-insertion into a lipid environment on Band 3 protein's circular dichroism spectrum and calculated secondary structure?

B. MATERIALS AND METHODS

1. Vesicle Preparation

The reconstitution method of Scheuring *et al.* (1986) was closely followed, but substituting the chemically homogeneous, and non-ultraviolet absorbing, detergent $C_{12}E_8$ for Triton X-100. For our application of extending the work of Scheuring *et al.* to conformationally sensitive spectroscopic measurements, it was an important substitution. Besides the minor inconvenience of interfering with the protein absorption monitoring of the Band 3 purification, the ultraviolet absorbance by Triton X-100 and possible impurities could interfere with the spectroscopic measurements. $C_{12}E_8$ is a nonionic detergent, with solubilizing potencies that are similar to those of Triton X-100 but, lacking the phenyl groups, it does not absorb appreciably at 280 nm. The partial specific volume (0.973 cm^3/g) and cmc (0.087 mM) of $C_{12}E_8$ are not very different from those of Triton X-100 ($\bar{\upsilon}$ =0.908, cmc=0.3 mM) (Reynolds, 1982) so it should be a suitable substitute for the vesicle formation. Importantly, we know that $C_{12}E_8$ does not alter BADS binding to Band 3 (Lieberman and Reithmeier, 1983).

In brief, the vesicle formation protocol was as follows. Dried egg yolk phosphatidyl choline (9 mg, Sigma Type V-E) was rehydrated with a solution of 1 mg of purified Band 3 in 0.1% $C_{12}E_8$, 50 mM sodium phosphate, pH 8 (see chapter II) in a total volume of 4.5 mL. This produces a lipid concentration of 2 mg/mL and an initial ratio of phospholipid to protein of 9:1 (wt/wt). The resulting milky suspension was then clarified by adding aliquots of a 10% stock of β-octyl glucoside. The clarification was monitored by measuring the apparent absorbance due to light scattering at 315 nm. Octyl glucoside was added until the absorbance was reduced to close to that of the initial buffer, and was not decreased upon further additions of octyl glucoside. The final concentration of octyl glucoside was generally between 0.7% and 0.9% (wt/vol). The step of monitoring the clarification was added to the Scheuring protocol, and it seemed to be a critical point in the procedure: it was noticed that if the clarification was not complete, there was substantially less protein incorporated into the vesicles. The solution was then dialyzed against 4 L of buffer A (10 mM Na_2SO_4, 10 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid, pH 7.2, 0.5 mM EDTA), at 4 °C for about 50 hrs, changing the dialysate 3 times (usually after 4, 18 and 30 hrs). Membrane association was checked by centrifugation of the sample at 70,000 rpm in a TLA 100.3 rotor for 30 minutes, and measuring the amounts of protein associated with the pellet and remaining in the supernatant. The pellet was resuspended in buffer A and the vesicles were purified by sucrose gradient centrifugation as described by Scheuring *et al.* (1986). The transluscent vesicle band was dialyzed against multiple changes of buffer A, pelleted at 70,000 rpm for 30 min, and resuspended in dH_2O at a protein concentration between 0.2 and 1.9 mg/mL. An estimate of the size of the vesicles was made using gel filtration on Sephacryl S-1000. The elution profile was broad and corresponded to an average diameter of 200 nm. This is larger than the values (100 nm) obtained by Schubert (1986).

Additional changes in the preparations used only for the CD measurements in collaboration with Dr. Jung were a reduction of the starting phospholipid/Band 3 ratio to

3:1 (wt/wt), and that the resulting vesicle suspension was frozen, thawed, and sonicated in a bath before the CD measurements.

2. BADS Binding Measurements

The measurements of BADS binding to Band 3 by fluorescence enhancement titration were performed as described in chapter II, using a normal viewing mode of 90°, and an excitation wavelength of 280 nm. BADS emission fluorescence was measured by virtue of energy transfer from Band 3 tryptophan residues to BADS in the stilbenedisulfonate binding site. In this way, fluorescence due to the interaction of BADS with the vesicles should be minimized. The background fluorescence of BADS in the presence of non-protein-containing vesicles was practically coincident with that in the absence of non-protein-containing vesicles up to 10 μM BADS (data not shown). Above 10 μM BADS, the dependence of the apparent intrinsic BADS fluorescence on the BADS concentration in the presence of vesicles was linear, while that in the absence of vesicles deviated from linearity, as expected from the effects of self-quenching. The background fluorescence in the presence of vesicles showed no apparent quenching, so corrections for self-quenching were not made for the Band 3-containing vesicle samples. The appropriate background fluorescence was subtracted from the measurements containing Band 3, using the background in the presence of vesicles to correct the measurements on Band 3-containing vesicles. Following correction for the background fluorescence, all fluorescence values were normalized for the measured protein concentration in the titration, and are presented as the fluorescence intensity per μg protein. One sample of Band 3 in $C_{12}E_8$ was sonicated prior to measuring BADS binding, using a probe sonicator at 100 watts for 30 seconds at 4 °C.

Double reciprocal plots of the binding data (1/F *vs.* 1/[BADS]) were linear in most cases, having linear regression correlation coefficients of 0.96 to 0.99. The maximum fluorescence, F_{max} (normalized for the protein concentration), and BADS concentration (mM) at half-maximal change in fluorescence, $C_{1/2}$, were determined from the double reciprocal plots. The values of F_{max} were quite variable, but the variability did not seem to be related to the type of sample. There was sometimes a slight downward curvature in the plots at high BADS concentrations, which may be attributed to the difficulty in making the correction for background fluorescence at high BADS concentrations. This may contribute to the variability in the F_{max} calculated from the best-fit line.

3. Analysis of Suspensions of Reconstituted Band 3 by Absorption and Circular Dichroism

The unpolarized absorption measurements were performed on a Cary 60 using the same cell (pathlength of 0.0103 cm) as for the CD measurements. Scans from 190 nm to 450 nm were taken of a suspension of Band 3-containing vesicles having 0.225 mg protein/mL (the same concentration as was used for the circular dichroism measurements), and of the same preparation after solubilization with 2.4 % (82 mM) octyl glucoside, correcting for dilution. The analysis of the absorption spectra is essentially the same as that done by Wallace and Mao (1984). The contribution of the components to the apparent absorption by the suspension as a function of wavelength (A_{susp} (λ)), is described by the equation: $A_{susp}(\lambda) = A(\lambda) + A_s(\lambda) - A_f(\lambda)$, in which A is the true absorption by the protein, A_s is the apparent absorption due to scattering, and A_f is the contribution of absorption flattening. The estimation of the scattering used only the values from the absorbance scan in the region of the spectrum where protein chromophore absorption is negligible (310 nm-400 nm), that is, where $[A(\lambda)] = A_f(\lambda) = 0$, so A_s is approximately equal to A_{susp}.

The absorption due to scattering (A_s) can be expressed as a power dependence of the wavelength (Wallace and Mao, 1984; Tanford, 1961), which is sufficiently represented by the approximation of a single power dependence on the wavelength (Wallace and Mao, 1984):

$$A_s(\lambda) = \Sigma k_i \lambda^{-n_i} \approx k \lambda^{-n}$$

where k_i and n_i are constants (n_i representing the power dependence). The linear form of this is used to determine the values of k and n:

$$-\log [A_s(\lambda)] = n \log (\lambda) - \log k$$

A linear least-squares regression analysis of $-\log [A_s(\lambda)]$ *vs.* $\log (\lambda)$ will have a slope equal to n. The values of k and n determined from the wavelength range 310 - 400 nm are then used to extrapolate to the region of the spectrum where protein chromophore absorption is significant, and where the circular dichroic bands arise. The contribution of scattering to the apparent absorption, extrapolated from the plot of $-\log[A_s(\lambda)]$ vs. $\log \lambda$, is subtracted from the measured absorbance of the suspension (A_s) to give $A_{susp} - A_s$. A technical note that should be mentioned is that the correction for the scattering component depends strongly on the slope (and thus on the choice of the line) for the plot of $-\log A_{susp}$ *vs.* $\log \lambda$. If the correction had been made from a plot in the region of the chromophore, which has a steeper slope and thus leads to a much larger correction, negative $A_{susp} - A_s$ would have resulted. This stresses the importance of estimating the correction from measurements outside the chromophore region.

Circular dichroism spectra without any correction for scattering effects were recorded on a Jasco J-500C spectropolarimeter, as indicated in chapter II, but using a 0.0103 cm pathlength cell, and a protein concentration of 0.225 mg/mL. The CD spectra using the scattered light collecting device (of which the pathlength was 0.0254 cm) were recorded by Drs. J.J. Chin and C. Jung as described in their recent publication (Chin *et al.*, 1987). Dilutions of the resuspended vesicles were made into dH_2O, with final concentrations of protein 0.140 mg/mL. This dilution of the vesicle suspension was determined to provide adequate ellipticity signal from the protein, and still maintain adequate light intensity. Protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1951), as modified by Peterson (1977), to avoid possible interference of the phospholipid with the protein determination.

Analysis of the CD spectra for the contents of secondary structure was done using three different analysis programs: a program based on Chen, Yang and Chau (1974), which was written by Dr. M. Ohnishi; a matrix analysis program (Compton and Johnson, 1986), provided by K. Oikawa; and the 1980 version of Contin, from the European Molocular Biology Laboratory (Provencher and Glöckner, 1981).

C. RESULTS AND DISCUSSION

1. BADS Binding to Reconstituted Band 3

The addition of phospholipid vesicles (non-protein-containing) to a solution of solubilized Band 3 had only a small effect on the BADS binding to Band 3. In the titrations shown in Figure III.1 the apparent maximal fluorescence intensity in the presence of phospholipid vesicles was somewhat higher at all concentrations of BADS, but the affinities of binding were similar, in both cases having a $C_{1/2}$ between 1 and 3 μM. The binding parameters derived from double reciprocal plots are listed in Table III.1. Measurement of BADS binding to Band 3 in solution in the presence of lipid vesicles serves as a control of the effect of lipid vesicles on the fluorescence enhancement titration. Although the presence of detergent in the assay suspension might affect the phospholipid vesicles, the concentration of detergent present was fairly low (≤0.005% $C_{12}E_8$, just at, or below, the cmc), and it was observed that the visible turbidity of the phospholipid vesicle suspension did not change upon addition of the $C_{12}E_8$-containing Band 3 solution. These results indicate that the presence of the large, light-scattering vesicles may slightly affect the fluorescence intensity that is detected per unit of Band 3-BADS. Nevertheless, the relative affinity of binding that is measured was essentially the same for Band 3 in the absence or the presence of phospholipid vesicles. The only difference noted was that slightly more curvature of the double reciprocal plots, perhaps indicating a heterogeneity of sites, was observed in the presence of the vesicles. This could result from imprecise correction for the background fluorescence, or be due to low affinity BADS binding to the lipid vesicles.

The saturation plot of the results of BADS fluorescence enhancement titration for one set of Band 3 reconstituted into vesicles is shown in Figure III.2. The binding parameters for 3 measurements on 2 preparations are listed in Table III.1. The results of BADS binding for Band 3 in vesicles were somewhat variable with respect to the F_{max} and $C_{1/2}$, but no more than those for Band 3 in $C_{12}E_8$ solution (with or without vesicles). As indicated by the F_{max} values, the fluorescence yield at maximal BADS binding was within the range of that in detergent, perhaps slightly higher. This suggests there are the same relative number of BADS binding sites per unit concentration of the Band 3 protein, i.e., that no binding sites have been lost upon reconstitution. The affinity of BADS binding by the Band 3 in vesicles was also normal, with one determination of $C_{1/2}$ slightly lower, and two slightly higher, than those in $C_{12}E_8$.

As was noted for the titrations using Band 3 in $C_{12}E_8$ plus lipid vesicles, there was a slight downward curvature of the double reciprocal plots at high BADS concentrations (though the fit to a linear equation was still reasonable), which might reflect the technical difficulty of background fluorescence subtraction at high concentrations of BADS. A double reciprocal plot of two of the titrations is shown to illustrate this effect (Figure III.3). We cannot rule out a very small component of binding that is of lower affinity than that represented by the best-fit line of the double-reciprocal plots. Although we cannot absolutely rule out some unusual effect of the presence of vesicles on the measurements, our results indicate that any such effect would be small, and that the binding parameters derived should validly represent the BADS binding properties of Band 3. The validity of using this fluorescence measurement in vesicle suspensions was only to be expected, since it was previously demonstrated that the results obtained using the large ghost membrane

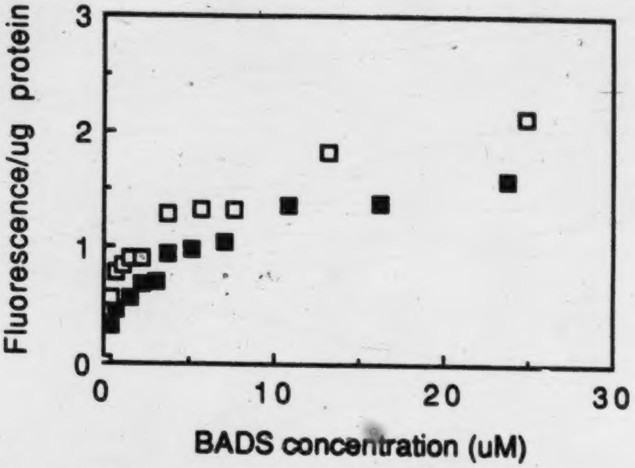

Figure III.1. BADS binding to Band 3 in the presence and absence of lipid vesicles. BADS binding to Band 3 was measured by fluorescence enhancement titration, as described in Methods, using an excitation wavelength of 280 nm and an emission wavelength of 450 nm. In this case, the Band 3 used in the titration had been purified by AE-Sepharose, and was in 0.1% $C_{12}E_8$, 20 mM sodium phosphate, pH 8, before dilution into the assay buffer. After 20-fold dilution (10 µL into 200 µL), the final $C_{12}E_8$ concentration would be 0.005%. The titrations were performed on this solubilized Band 3 either with no other additions (■), or with the addition of lipid vesicles (□) (adding the same volume as of the reconstiuted Band 3), which had been prepared as described for the Band 3-containing vesicles, but without protein. The background fluorescence of BADS was subtracted from each set of data before graphing.

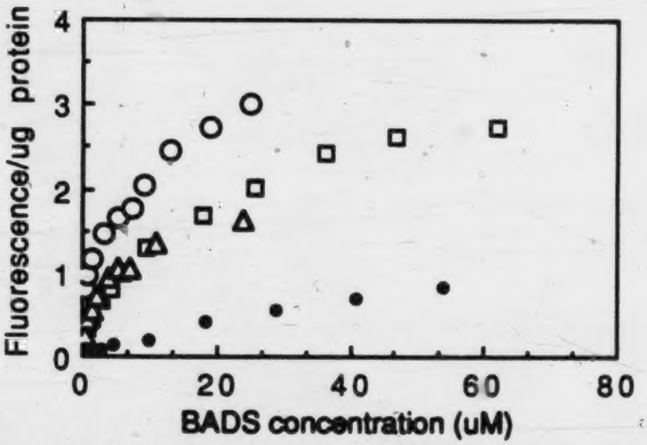

Figure III.2. BADS binding to reconstituted Band 3. A fluorescence enhancement titration of BADS binding to two samples of reconstituted Band 3 (□,○) was measured. BADS binding to Band 3 in $C_{12}E_8$, similar to that shown in Figure III.1 is included for comparison (△). The background fluorescence due to BADS (●), determined in the presence of lipid vesicles, had been subtracted from the data prior to graphing. Fluorescence intensities have been normalized for the protein concentration in the titration.

TABLE III.1.
BINDING PARAMETERS FROM BADS FLUORESCENCE ENHANCEMENT TITRATIONS WITH BAND 3 IN DETERGENT AND IN PHOSPHOLIPID VESICLES

sample	$C_{1/2}$ (μM)	F_{max} @
Band 3 in $C_{12}E_8$ (sonicated) *	1.4	1.2
Band 3 in $C_{12}E_8$ §	2.7	2.6
Band 3 + PL vesicles *	1.3	1.5
Band 3 + PL vesicles	2.4	1.5
Band 3 in vesicles (sample B, sonicated) §	0.8	2.1
Band 3 in vesicles (sample B)	3.7	1.9
Band 3 in vesicles (sample A)	3.4	2.3

* data shown in Figure III.1

§ data shown in Figure III.2

@ fluorescence was normalized for the concentration of protein in the assay before determination of F_{max} and $C_{1/2}$.

fragments compared well with the K_i of BADS in cells (Lieberman and Reithmeier, 1983). However, the phospholipid/Band 3 ratio is lower in the ghost membranes (about 4:1 (wt)) than in the vesicles (starting ratio was 9:1 (wt)), so lower amounts of ghost membranes were necessary to perform a measurement, and the visible turbidity was much less than

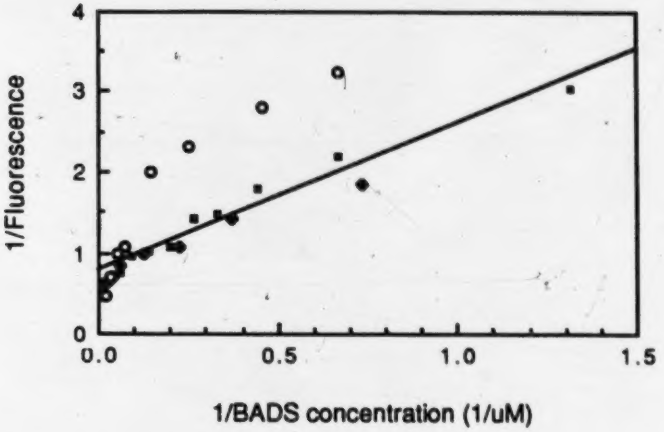

Figure III.3 Double reciprocal plot of BADS binding to Band 3. BADS binding was measured as described in Figures III.1 and III.2. The data were normalized for the concentration of protein in the assay. The titrations shown are for Band 3 in $C_{12}E_8$ (■), Band 3 in $C_{12}E_8$ plus lipid vesicles (◆), and Band 3 in $C_{12}E_8$ that had been exposed to 2% OG (○) prior to the BADS binding measurement. The linear regression-fitted line for only one set of data (■) is shown.

when using the Band 3-containing vesicles.

It is interesting to note that the dramatic affect of octyl glucoside on the BADS binding by Band 3 that was noted in Chapter II was reproducible using the preparation of Band 3 that was used for formation of vesicles. Addition of octyl glucoside to the Band 3 in 0.1 % $C_{12}E_8$ (to 2%, about 71 mM) resulted in a distinctly two-component BADS binding behavior (Figure III.3). The minor component had a $C_{1/2}$ = 1.6 µM and F_{max} = 8.6 (compared to F_{max} = 28.5 for the same amount of protein in $C_{12}E_8$). The major component had a $C_{1/2}$ >20 µM and an F_{max} of 87 (fluorescence units). The data presented in Chapter II were qualitatively similar, but only a single component of reduced affinity (11 µM) was detected.

To put this in perspective with the transport studies done by Scheuring *et al.*, recall that they noted altered inhibition characteristics of several inhibitors (Scheuring *et al.*, 1986, p. 130). They found that the concentrations of inhibitors producing 50% inhibition

($C_{1/2}$) of sulfate efflux were fairly close to the respective K_i values obtained in red cells: $C_{1/2}$ for H_2DIDS was 0.1-0.2 μM (in 10 mM Na_2SO_4), which is close to the K_i of 0.05 μM (Knauf, 1979); $C_{1/2}$ of 2.0 μM for DNDS and 0.7 μM for flufenamate represent a 4 x increase, and 7 x decrease, respectively, from values in erythrocytes. However, above the K_i,the increase in inhibition with increasing inhibitor concentrations was less than expected for a simple 1:1 interaction of the inhibitor with a uniform population of Band 3 molecules (Scheuring *et al,* 1986.). A similar result was observed by Köhne *et al.* (1983) in transport measurements on their reconstituted preparation. The interpretation in the Schubert report of the non-stoichiometric inhibitor binding beyond the K_i is that part of the Band 3 molecules in the vesicle membrane may have undergone slight conformational changes.

Our BADS binding experiment indicates that, although there could be a small component with lower-affinity BADS binding, the majority of the Band 3 in the vesicles retains normal BADS binding affinity. We thus have measurements indicating normal BADS binding affinity for the major steps of the reconstitution procedure: Band 3 in the erythrocyte membrane; Band 3 purified in $C_{12}E_8$; and the reconstituted Band 3. The only step that has not been monitored is the Band 3,$C_{12}E_8$,OG,egg phosphatidylcholine mixture prior to dialysis, but the finding of normal binding in the vesicles makes this value less important. The effect of added OG on the BADS binding of Band 3 is dramatic compared to the binding characteristics of the Band 3-containing vesicles, making it clear that most of the detrimental effect of OG is prevented in this reconstitution procedure. We are uncertain as to what results in the unusual reduction in inhibitor potency detected in the transport studies of Schubert. It could result from a partial denaturation of some of the Band 3 molecules in the vesicles. It is also possible that there may be lipid effects on the inhibitor binding, as has been noted for the glucose transporter from human erythrocytes (Hah, Hui and Jung, 1983), that are not present in the native membrane. Or they could be due to non-

Band 3-specific action of the inhibitors, which would be minimized in our binding measurements.

2. Circular Dichroism Measurements on Reconstituted Band 3

Wallace suggests that the CD spectral artifacts are minimal for proteins in membrane preparations of small unilamellar vesicles (Wallace and Mao, 1984; Mao and Wallace, 1982). The Band 3 vesicle preparation had been reported (Schubert *et al.*, 1986) to result in vesicle sizes that are larger than what is considered small vesicles (about 70 nm diameter), and we found our preparation was probably even larger than in Schubert's report. We therefore wanted to estimate the severity of the contribution of the light scattering and absorption flattening artifacts in our preparation of Band 3-containing vesicles.

a. Estimation of Light Scattering and Absorption Flattening. As a first step, estimates of the contributions of light scattering and absorption flattening to the unpolarized absorption spectra of the Band 3-containing vesicles were determined. The methods of analysis are covered in the Methods section. Essentially, we followed the analysis described by Wallace and Mao using bacteriorhodopsin (1984). The estimate of the contribution of scattering to the absorption spectrum employs measurements of the unpolarized absorption spectra of the suspension of Band 3-containing vesicles (A_{susp}). Because the measurements are in a region of the spectrum where protein chromophore absorption is low, the apparent absorption is due to scattering of the light . We have analyzed the unpolarized absorption spectrum of the Band 3-containing vesicles in the wavelength region of 230 to 400 nm and found a power dependence (n) of 4.23, with a correlation coefficient of 0.99 (Figure III.4). Note, in Figure III.4B that the dependence varied outside of this wavelength range. The theoretical value of n, for a model of small, solid, spherical particles of diameter $< 1/20\ \lambda$, should be 4.0 (Wallace and Mao, 1984). Examples of experimental power dependencies of membranous samples found by Wallace

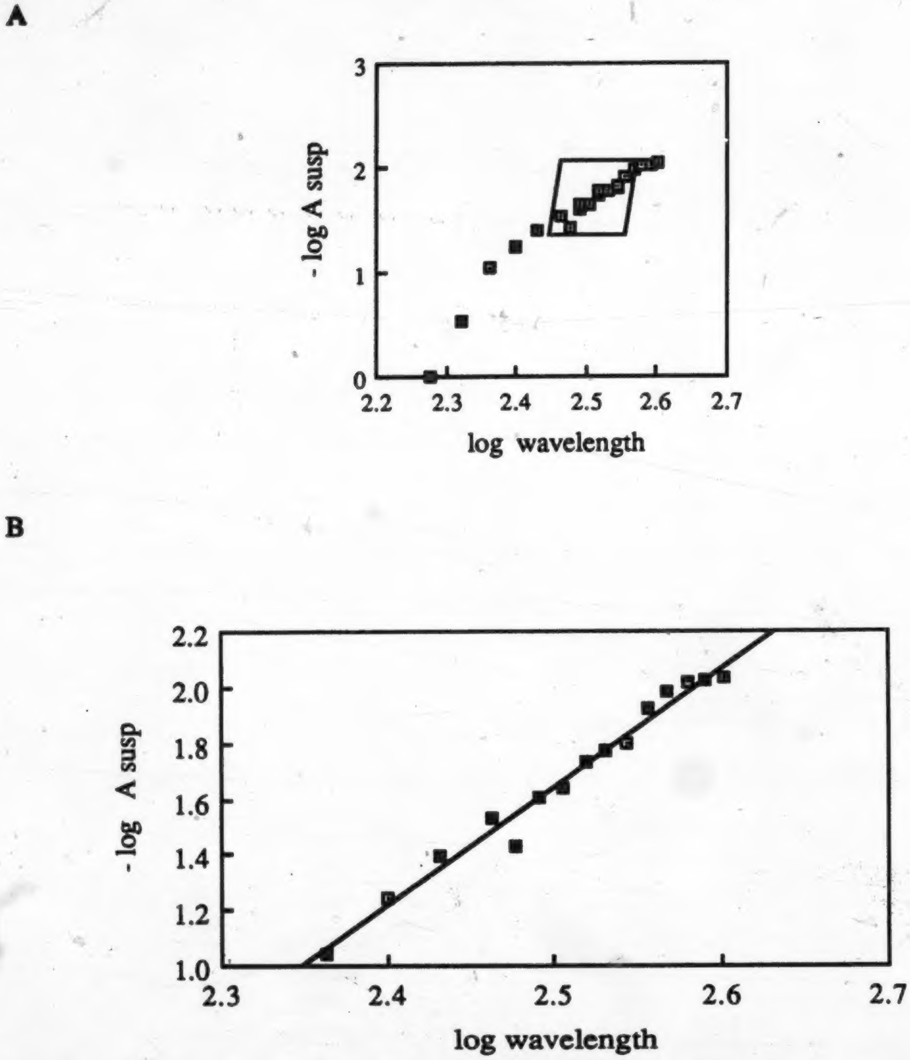

Figure III.4. Plots of -log [$A_{susp}(\lambda)$] *vs*. log (λ) for Band 3 in lipid vesicles. An absorption spectrum of a suspension of Band 3-containing vesicles at a protein concentration of 0.225 mg/mL, in diluted buffer A, was measured in a cell of pathlength 0.013 cm (the same cell as used for the CD measurements). This graph represents the linear form of the single power dependence of the absorption due to scattering on the wavelength, as given by: $-\log [A_s (\lambda)] = n \log (\lambda) - \log k$.

A. Spectrum over a wide wavelength range (190 - 450 nm). The region enclosed by a parallalogram represents the wavelength range of 310-400 nm that is expected to be suitable for determination of the the power dependence, n (and which is represented in B).

B. The region of the spectrum where protein absorption is low (310 nm to 400 nm), as well as through the aromatic absorption region (230 to 310 nm, log (310) ≅ 2.5). The line shown was fit by linear regression to the equation : $y = -8.93 + 4.23\ x$, with a correlation coefficient of 0.99.

and Mao are: about 4.4 for small unilamellar vesicles without proteins, 3.2 for purple membranes, and 3.4 for membrane sheets containing Na/K-ATPase (Gresfali and Wallace, 1984). Our value is within this range.

The unpolarized absorption spectra are shown in Figure III.5. The ultraviolet absorption spectrum (190-240 nm) of the Band 3-containing vesicles (A_{susp}) is reduced in intensity at the lower wavelengths, compared to the detergent-solubilized preparation (A_{sln}), and has a higher intensity at the higher wavelengths, compared to solubilized. Octyl glucoside-solubilized is a suitable reference in this case, since unpolarized far ultraviolet absorption spectra are relatively insensitive to protein conformation (Rosenheck and Doty, 1961). After correction for the estimated component due to scattering ($A_{susp} - A_s$) the part of the spectrum at the higher wavelengths (220 to 240 nm) is almost coincident with that of the solubilized vesicles (A_{sln}). At the lower wavelengths, however, the reduction in the absorption intensity for this corrected spectrum of the suspension is even more apparent than before the scattering correction. This indicates that there is significant absorption flattening in the spectra of this suspension of Band 3-containing vesicles. The presence of absorption flattening effects may give rise to differential absorption flattening effects in the circular dichroic spectrum.

The degree of differential scattering may be quite different than that of the scattering of unpolarized light, making it difficult to estimate the relative severity of scattering. artifacts. There is definitely light scattering caused by the sample, since the scattering correction from the unpolarized ultraviolet absorbance was of the order of 0.1 to 0.2 absorbance units at the protein concentrations used. The wavelength dependence of the scattering effect was closer to that of small unilamellar vesicles than of membrane sheets, suggesting the effect may be minimal.

b. CD spectra of Reconstituted Band 3. Spectra were obtained on an instrument without the capabilities for changing the detector geometry, so measurements at

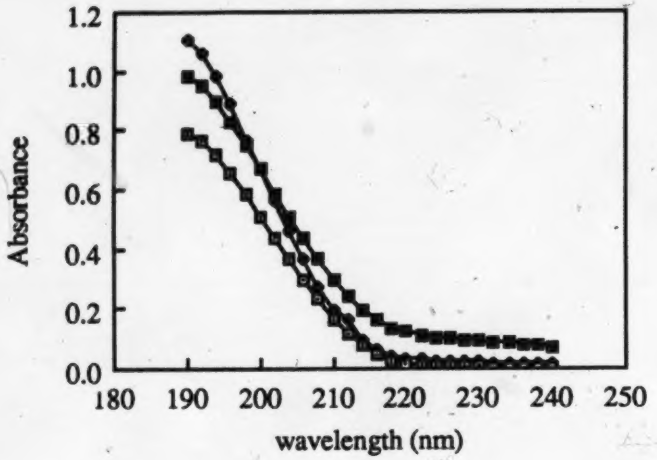

Figure III.5 Absorption spectra of Band 3 in lipid vesicles. $[A_{susp}(\lambda)]$ (-■-), of vesicles solubilized in 2.3% OG $[A_{sln}(\lambda)]$ (-◇-), and the calculated spectrum for the vesicles following correction for scattering $[A_{susp}(\lambda) - A_s(\lambda)]$ (-▣-). The measurements were made in a 0.013 cm pathlength CD cell, in diluted buffer A. The protein concentration for the suspension was 0.225 mg/mL. The data shown for the solubilized spectra have been corrected for the dilution caused by the addition of detergent .

variable detector acceptance angles which minimize the scattering effects, were not possible. The only precautions taken to minimize the effects of scattering were to use short pathlength cells. The resulting spectra were severely distorted (not shown). Even the "reference-state", here represented by octyl glucoside-solubilized reconstituted Band 3, was unusually shaped, and more importantly, of much lower intensity than expected. An attempt was made to correct for artifacts due to absorption flattening, using the flattening quotient method described by Mao and Wallace (1984), however, the approach was found not suitable[1] for these spectra, and OG-solubilized Band 3 may not be a suitable reference state. It is possible that protein solubilized in 2.3% OG does not provide a good reference state, even though we had found previously that OG (up to 60 mM) did not reduce the intensity of the CD bands at 208 and 222 nm. Perhaps $C_{12}E_8$ might provide a more suitable reference solubilized state. Such severely distorted spectra are of little use, and it appears necessary to be able to collect spectra in which the contribution of differential scattering is minimized.

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[1] In the "corrected" spectrum the value of mean residue ellipticity at 190 nm, a wavelength that is very susceptible to the artifacts, is not increased to that found in the OG-solubilized sample

To obtain a CD spectra of the Band 3-containing vesicles that is free from the distorting effects of differential light scattering, spectra were measured using a scattered-light collecting device that had been designed by Dr. Chan Jung and co-workers (Chin *et al.*, 1987). They had designed this ingenious light reflection device for their studies on proteoliposome vesicles of the erythrocyte glucose transporter. The Band 3-vesicle preparation that was used for the measurements using this device was slightly different from the preparations used for BADS binding, the unpolarized absorption measurements, and the other CD measurements in that it had a higher protein/phospholipid ratio. It was anticipated that this would reduce the scattering effects for a given amount of protein chromophore.

The spectra obtained by Drs. J. Chin and C. Jung are shown in Figure III.6. These are single scans, and represent the best spectrum of reconstituted Band 3 thus far obtained. The shape of the spectrum appears free from obvious distortions, in that its shape is similar to that for detergent-solubilized Band 3, and the ratio of $[\theta]_{190}$ to $[\theta]_{222}$ is normal, or a bit higher than expected (about 3). However, inspection of the molar ellipticities indicates unusually high values, approaching those of the reference spectrum for the α-helical conformation (Chen *et al.*, 1972). This is not consistent with the values obtained for the solubilized protein in any detergent.

Nevertheless, the spectra were analyzed for an estimate of secondary structural content, using three different analysis programs. The values obtained for the Band 3-containing vesicles will be discussed. A program based on Chen, Yang and Chau (1974),

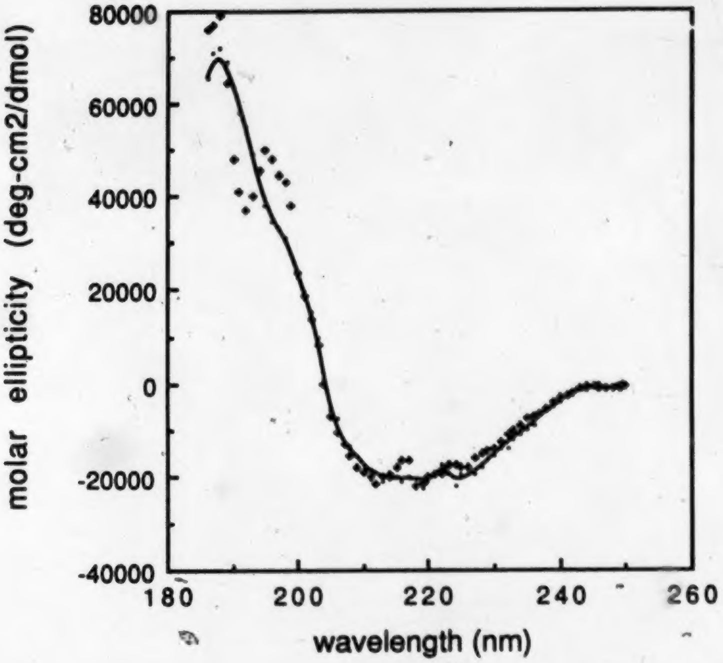

Figure III.6 Circular dichroism spectra of reconstituted Band 3 in the absence and presence of DIDS. The spectra were run using a scattered light-collecting device, and represent single scans. The vesicle suspension was diluted into dH_2O, resulting in a protein concentration of 0.14 mg/mL (—). DIDS was added to a concentration of 10 μM (+) to this same dilution of reconstituted Band 3.

which includes a helix length-dependence, produced values of α-helix varying from 89% to 67%, using a helix length of 10 to 25 residues, respectively. A length of about 10 residues is normal for globular, soluble proteins. Membrane proteins, however, may have a longer average helix length, since it takes approximately 21 residues in a perpendicular α-helix to cross the hydrophobic core of the bilayer. This analysis, although constrained to have a sum of all components of 1.0, produced negative values for β-sheet. Therefore, the estimate of α-helix may be an overestimate. Using the matrix analysis program of Compton and Johnson (1986) a value of 65% helix was obtained The unconstrained sum of components was -0.81. This value, for a good fit, should be 1.0, suggesting a poor fit

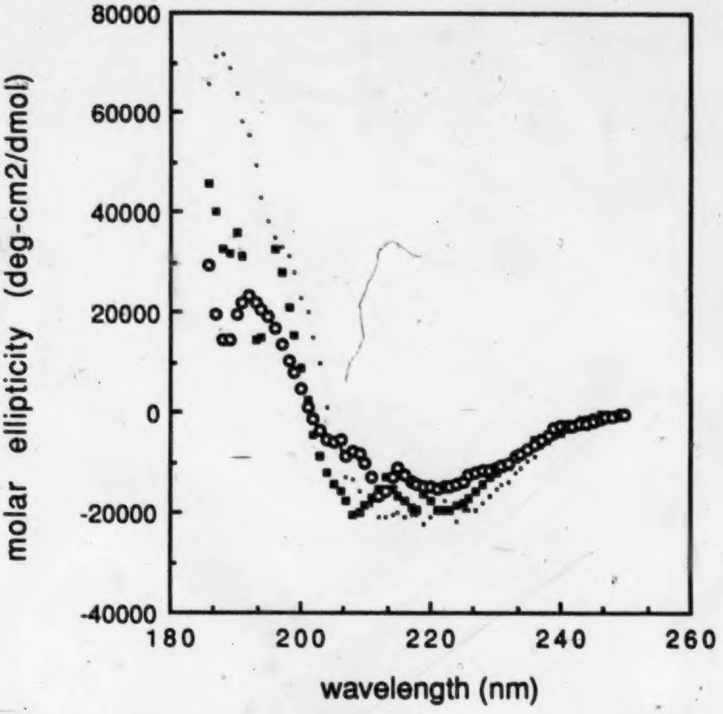

Figure III.7 Circular dichroism spectra of reconstituted Band 3 after solubilization with detergents. The spectra were run using a scattered light-collecting device, and represent single scans. The vesicle suspension was diluted into dH_2O containing 0.2% of either $C_{12}E_8$ (■), or octyl glucoside (○), resulting in a protein concentration of 0.14 mg/mL. The spectrum of the Band 3 vesicles prior to solubilization (·) is included for comparison.

of this data for the components other than helix. The value for helix is, however, generally reliable. The best fit chosen by the program Contin (Provencher and Glöckner, 1981) was 100% α-helix. This further illustrates that the α-helical content represented in this spectrum is unexpectedly high, for intact Band 3, in comparison to the previous measurements in several detergents. The wide variation in the helix content predicted by the different programs suggests that this spectrum must be unusually shaped compared to the basis spectra. Whether the α-helical content of Band 3 in vesicles is actually higher than in detergent-solubilized state remains to be shown, and will require further experimentation.

Comparison of the spectra for different conditions may be more reliable. Addition of 10 µM DIDS to the Band 3-vesicle suspension produced an essentially identical spectrum to that without DIDS. The finding of no change in the net secondary structure with the addition of this inhibitor supports earlier results using Band 3 in $C_{12}E_8$ (Oikawa *et al.*, 1985). The addition of detergents may be less comparable, since the particulate nature of the sample is being altered. Solubilization of the Band 3-containing vesicles with 0.2% $C_{12}E_8$ produced very little change in the spectrum, while solubilization in 0.2% octyl glucoside caused an apparent reduction in the magnitude of ellipticities around both the 220 nm and the 190 nm region (Figure III.7). This means that the ellipticities are still unusually large, when comparing to those of $C_{12}E_8$-solubilized Band 3 using a normal cell. The spectrum in OG, and the values of helix calculated from it, is similar to that obtained previously by us (Werner and Reithmeier, 1985; and Chapter II). Future studies should clarify the differences between the CD spectra obtained, using a scattered light-collecting device, for reconstituted Band 3, and those obtained for detergent solubilized Band 3.

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IV. ONE MECHANISM OF INHIBITION OF ANION EXCHANGE BY A MEMBRANE-IMPERMEANT CARBODIIMIDE: INHIBITION IN CITRATE-CONTAINING MEDIA[1]

A. INTRODUCTION

The 95,000 dalton integral membrane protein of human erythrocytes, known as Band 3 protein, functions to facilitate the exchange of anions across the membrane (for a recent review, see Passow, 1986). The amino-terminal cytoplasmic domain (Mr=41,000) appears not to be necessary for the transport function of Band 3 (Lepke and Passow, 1976; Grinstein, Ship and Rothstein, 1978), while the carboxyl terminal domain (Mr=55,000) spans the membrane bilayer at least eight times (Passow, 1986; Jennings, Anderson and Monaghan, 1986) and contains the segments required for anion exchange (Grinstein, Ship and Rothstein, 1978). The publication of the sequence of the murine anion exchange protein, determined from the sequence of a cloned cDNA (Kopito and Lodish, 1985a), has allowed comparison of the many results from proteolysis and chemical modification, and has resulted in several models of folding of the protein (Kopito and Lodish, 1985b; Jay and Cantley, 1986; Passow, 1986). Identification by chemical modification of several different amino acid residues essential to or involved in anion exchange has made possible investigations of the relationships between structure and function in the human protein. Evidence for the involvement of arginine residues, believed to contribute to the anion binding site (Falke and Chan, 1986), is substantial (Passow, 1986; Bjerrum, Wieth and Borders, 1983). At least two lysine residues (denoted Lys *a* and Lys *b*), although apparently not directly involved in anion translocation (Jennings, Monaghan, Douglas and

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[1] **A preliminary report of this work has appeared: Werner, P.K. and Reithmeier, R.A.F. (1987) *Biophys. J.* 51, 513a, and a version of this Chapter has been published: Werner, P.K. and Reithmeier, R.A.F. (1988) *Biochim. Biophys. Acta.* 942, 19-32.**

Nicknish, 1985), have been defined by their reactivity with several inhibitors of anion exchange, most notably H_2DIDS.

The involvement of carboxyl residues in the anion exchange function was suggested by the pH dependence of exchange flux (Wieth and Bjerrum, 1982; Milanick and Gunn, 1982; Milanick and Gunn, 1984), in which deprotonation of a group with an apparent pK_a of 5 on the outside of cells was shown to be required for monovalent anion exchange, and protonation of a group of similar pK_a was shown to activate Band 3-mediated sulfate influx (Milanick and Gunn, 1984). The value and low temperature dependence of the pK_a indicated the titratable group is likely a carboxyl group (Wieth *et al.*, 1982a). The postulated involvement of carboxyl residues is supported by chemical modification studies, using carbodiimides as carboxyl-selective reagents to inhibit transport (Deuticke, 1977; Wieth *et al.*, 1982a; Wieth *et al.*, 1982b; Andersen *et al.*, 1983; Bjerrum, 1983; Craik and Reithmeier, 1984; Craik and Reithmeier, 1985). Using another carboxyl reagent, Woodward's Reagent K, Jennings and Anderson (1987) recently provided evidence that modification of at least two glutamate residues results in transport inhibition.

The previous work from our laboratory (Craik and Reithmeier, 1985) indicated that the characteristics of the inhibition by the membrane-impermeant carbodiimide ETC[2] appeared to be sensitive to the reaction medium: reaction in citrate-buffered sucrose produced irreversible inhibition, and reaction in phosphate-buffered saline produced reversible inhibition (i.e., inhibition remained after washing away the ETC, but reversed upon prolonged incubation of 60 minutes or longer). The differing inhibition characteristics were proposed to be due to the difference in orientation of the anion binding site of the protein in the two reaction media (Craik and Reithmeier, 1985). However, it was not determined which residues are modified to result in inhibition of anion exchange, or what accounts for the difference in inhibition in the two conditions. The work described herein shows that the inhibition in the two media appears to arise by different mechanisms. Specifically, we present evidence that the inhibition produced by ETC in citrate-containing medium is due to carbodiimide activation of citrate and subsequent modification of a residue (possibly lysine *a*) in Band 3. The inhibition produced in saline results from a different mechanism, and is the topic of the following chapter.

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[2] This is an alternate nomenclature for that sometimes used for the same compound, 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide (EAC).

B. MATERIALS AND METHODS

Carrier-free [^{32}P]phosphate and [1,5-^{14}C]citric acid (56.9 mCi/mmol) were obtained from New England Nuclear. 1,2-ditritio-1,2-(2,2-disulfo-4,4-diisothiocyano) diphenylethane ([^{3}H]H_2DIDS, at 202 mCi/mmol) was obtained from Research Development Corporation, Hospital for Sick Children, Toronto. DIDS and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were from Pierce Chemical Company, Rockford Illinois. BADS was synthesized according to Kotaki *et al.* (Kotaki, Naoi and Yaki, 1971) as previously described (Rao *et al.*, 1979). 1-ethyl-3-[3-(trimethylammonio)propyl] carbodiimide iodide (ETC) was prepared from the free base of 1-ethyl-3-(3-dimethylamino propyl) carbodiimide by the method of Sheehan *et al.* (Sheehan, Cruickshank and Boshart, 1961), as described previously (Craik and Reithmeier, 1985). Outdated human red cells were kindly donated by the Canadian Red Cross. All other chemicals were reagent grade or better from standard suppliers.

1. Preparation of Erythrocytes. Red cells were washed four times with 10-15 volumes of 0.9% w/v NaCl at 4 °C to remove storage medium and white cells. If chloride-free medium was required, the cells were then washed 3 times with 10 volumes of citrate-buffered sucrose (28.5 mM sodium citrate, pH 7.0, 205.3 mM sucrose).

2. Treatments of Cells. The usual conditions for treatment with ETC in citrate-buffered sucrose were as follows: ETC was added to a final concentration of 4.5 mM (by dilution from a freshly dissolved solution in the reaction buffer) to a 50% suspension of cells in 28.5 mM citrate, 205.3 mM sucrose, at pH 6.8, and the suspension was incubated at 37 °C for 5 minutes. After the incubation with ETC, the cells were then washed twice with ice-cold reaction buffer containing 0.5% bovine serum albumin and three times in the buffer to be used for the next incubation or for transport measurements. In some cases, treatment was done similarly, but with changes in ETC concentration or the buffer composition that are mentioned with each particular result. In the series of citrate-buffered sucrose solutions the calculated osmolarity was maintained relative to that containing 28.5 mM citrate by the addition of sucrose. Treatment of cells with α-chymotrypsin was done at 1 mg/ml enzyme for 1 hr at 37 °C at a 25% suspension of cells in PBS.

3. Transport Measurements. The exchange of extracellular phosphate for intracellular chloride was measured by following the loss of $[^{32}P]$ phosphate from the supernatant of a 50% suspension of chloride-loaded cells in 28.5 mM citrate-buffered sucrose, pH 7.4, at 30 °C, as described previously (Ho and Guidotti, 1975; Craik and Reithmeier, 1985; Craik and Reithmeier, 1986). Inhibition of transport was estimated from the change in half time for the initial loss of radioactivity from the extracellular medium. Inhibition (%) = $(1 - (t_{1/2}\text{ control} / t_{1/2}\text{ treated})) \times 100\%$. Transport measurements at higher external concentrations of phosphate were performed by addition of appropriate volumes of $[^{32}P]$phosphate from the same stock, and citrate-buffered sucrose to make a 50% suspension of cells. The anion flux (mM/min), which is expressed per unit phosphate concentration for the same volume of cells, was calculated from the initial rate of loss of $[^{32}P]$ from the supernatant, and the initial external phosphate concentration.

4. Preparation of Erythrocyte Membranes. Following post-treatment washing, cells were lysed in ice-cold 5 mM sodium phosphate, pH 8, containing 0.1 mM phenylmethylsulfonyl fluoride (Dodge, Mitchell and Hanahan, 1963), and washed extensively with the same buffer. An exception is the $^3H_2$DIDS crosslinking experiments, in which ghost preparation was done at pH 7.0. For the measurements of incorporation into membranes of either $[^{14}C]$citrate or $^3H_2$DIDS, aliquots of a diluted suspension of membranes were taken for scintillation counting, protein determination, and gel electrophoresis. Ghosts were stripped of peripheral proteins by treatment with 20 volumes of ice-cold 2 mM EDTA, pH 12, for 20 minutes. Chymotrypsin treatment of ghosts involved exposure of a 1:4 suspension of ghosts in 5 mM sodium phosphate, pH 8.0 to 250 µg chymotrypsin per mL for 1 hr at 37 °C. Digestion was terminated by addition of phenylmethylsulfonyl fluoride to 0.5 mM, and the ghosts were washed in 5 mM sodium phosphate, pH 8.0.

5. [^{14}C]Citrate Labeling. A stock of citrate-buffered sucrose was prepared by dissolving lyophilized [1,5-^{14}C]citric acid in 5 mM citrate, 285 mM sucrose, pH 7, resulting in a specific radioactivity of 197,300 dpm/nmol citrate (89.7 nCi/nmol). Erythrocytes washed in 5 mM citrate buffered sucrose were suspended at 50% suspension in the [^{14}C]citrate buffered sucrose and treated with 4.5 mM ETC as described above. Treatments in media containing 1 mM and 28.5 mM citrate were done similarly, at specific radioactivities of 107,000 dpm/nmol for 1 mM, and 19,000 dpm/nmol for 28.5 mM citrate.

6. Covalent ^{3}H$_2$DIDS Labeling. Solid ^{3}H$_2$DIDS (202 mCi/mmol) was dissolved in PBS (150 mM NaCl, 5 mM sodium phosphate), pH 7.0, at a concentration of 240 µM, and was stored in aliquots at -20 °C. Cells washed in PBS were treated at 25% suspension with 15-50 µM ^{3}H$_2$DIDS, without dilution with nonradioactive H$_2$DIDS, for 90 min at 37 °C (Shami, Rothstein and Knauf, 1978). For bifunctional reaction the treatment was done in 150 mM NaHCO$_3$, pH 9.5, for 90 min at 37 °C (Jennings and Passow, 1979). To remove reagent not covalently bound to the membranes, the cells were then washed three times with PBS containing 0.5% fatty acid free bovine serum albumin, then with buffer without added albumin.

7. Noncovalent Binding of ^{3}H$_2$DIDS. The reversible binding of ^{3}H$_2$DIDS to ghost membranes was performed under conditions similar to those described by Shami *et al.* (Shami, Rothstein and Knauf, 1978), with the special precautions to reduce covalent binding by using a low temperature (4 °C) and short times of exposure (5 min or less).

Measurements were similar to those described for [^{14}C]BADS binding (Lieberman and Reithmeier, 1983), separating the membrane-bound from the free $^3H_2$DIDS by centrifugation for 1/2 minute at about 100,000 x g (4 minutes elapsed time) in an air-driven ultracentrifuge with a pre-cooled titanium rotor. The covalent component of the binding was estimated by thorough washing of the membranes remaining after removal of aliquots for the noncovalent measurements, and processing as described above for covalent $^3H_2$DIDS labeling. A measurement of the nonspecific component of the noncovalent binding was attempted by using membranes that had been treated with 20 μM unlabeled DIDS, or by inclusion of 27 μM unlabeled DIDS in the incubation with $^3H_2$DIDS.

8. BADS Binding. Binding of BADS to Band 3 in ghost membranes by fluorescence enhancement titration was performed as previously described (Rao *et al.*, 1979; Lieberman and Reithmeier, 1983; also see Chapter II Methods), using an emission wavelength of 450 nm and excitation wavelengths of 340 nm or 280 nm. Binding parameters were estimated from double-reciprocal plots (1/F *vs.* 1/[BADS]) of the data.

9. Analytical Techniques. SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970) and protein bands were stained with Coomassie Blue. Densitometry of Coomassie Blue-stained gels was performed on a Joyce-Loebl Chromoscan 3 at 530 nm. The protein concentration was determined according to Lowry *et al.* (1951), in the presence of 1% SDS, using bovine serum albumin as a standard. Fluorography involved pretreatment of the gel with Enlighten enhancer (New England Nuclear), and exposure of the dried gel at -70 °C to Kodak X-Omat X-ray film. Calculation of the distribution of radioactivity in gels was done either by densitometry of a fluorogram prepared using pre-flashed film, or by scintillation counting of gel slices that had been digested by addition of 0.4 ml H_2O_2 and heating for 16 h at 50 °C. Scintillation counting was performed in a Beckman LS7800 scintillation counter with an external standard method of quench correction.

C. RESULTS

1. Characterization of the Inhibition

Treatment of erythrocytes in citrate-buffered sucrose with the carbodiimide ETC resulted in irreversible inhibition of anion exchange. The usual conditions for treatment (as detailed in the Methods section, with 4.5 mM ETC at pH 6.8 for 5 min) resulted in, on average, 60% inhibition of anion exchange, assayed as $^{32}P_i$ influx into chloride-loaded cells at low external phosphate concentrations (6-8 mM). This level of inhibition is described as "irreversible" since, besides remaining after washing away the treatment medium, additional incubation in the absence of reagent did not reduce the level of inhibition. This is in contrast to the reversible inhibition produced in PBS (which is the subject of the following chapter).

Attempts to increase the inhibition, or the amount of modification, by using higher concentrations of ETC, a higher pH, and longer times of treatment, rarely produced higher than 60% inhibition (data not shown). For example, a 50% suspension of cells in 28.5 mM citrate-buffered sucrose was treated with 20 mM ETC at pH 9.5 for 30 minutes, or first incubated at pH 6.5 for 3 minutes, then adjusted to either pH 9.5 (for 60 minutes more), or pH 10.5 (for 5 minutes). These treatments produced at most a 58% inhibition of transport, which was accompanied by a 77% loss of $^3H_2$DIDS covalent labeling sites (0.50 nmol $^3H_2$DIDS/mg membrane protein remaining). The reports of up to 80% inhibition by Craik (Craik and Reithmeier, 1985; Craik, unpublished) were not achieved, providing that the control cells were exposed to the same incubation conditions.

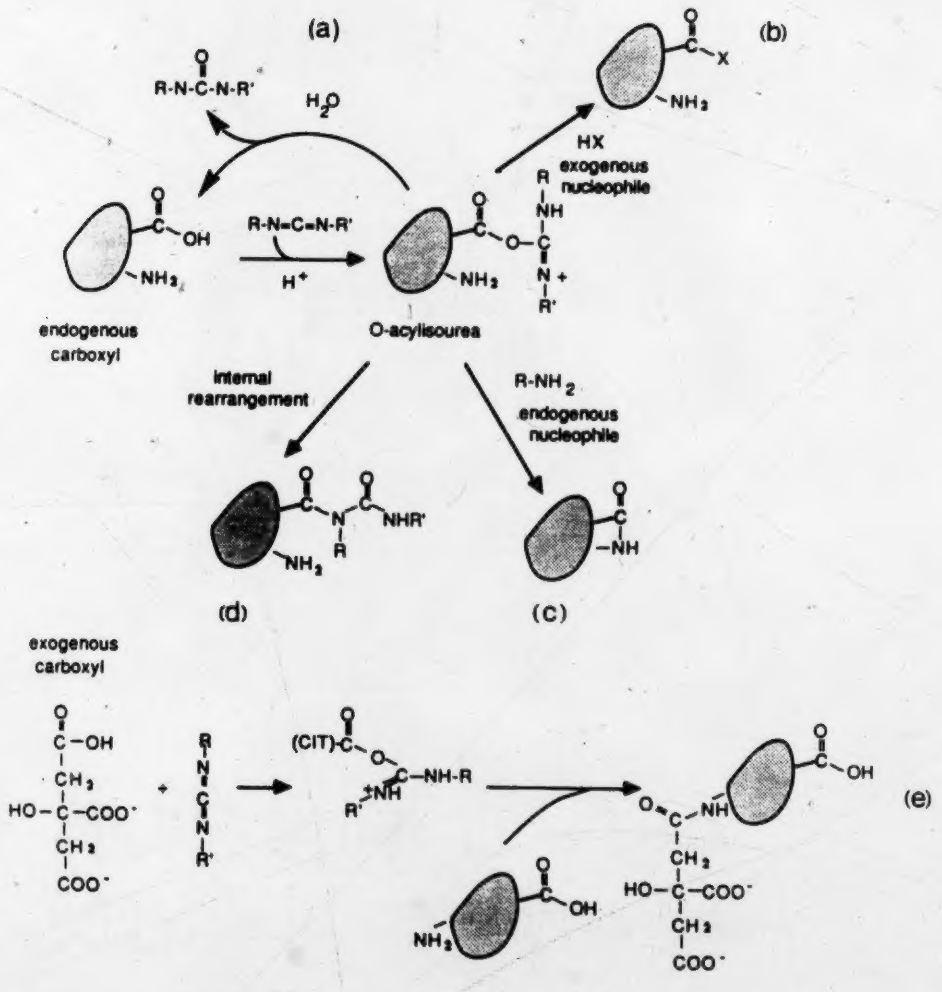
In addition to the usual measurement of transport, in which the limiting external anion is in low concentration, transport measurements at high concentrations of external phosphate were also performed. Such a study should potentially be able to distinguish between effects of this inhibitory treatment on the affinity for substrate anions (K_s), as opposed to an alteration in the maximal transport rate (V_{max}, in analogy to enzyme kinetics). In the ideal cases of 1) only an effect on the V_{max} , or, 2) only an effect on the

K_s , the expected results would be: 1) a constant percentage inhibition over varying substrate (P_i) concentrations, and 2) diminishing inhibition at high, close to saturating, substrate concentrations. Transport measurements were performed in concentrations of [^{32}P]phosphate from 5 mM to 189 mM, and the anion flux, which is expressed per unit phosphate concentration, was calculated (see Methods). The level of inhibition by ETC was slightly, but not dramatically, reduced when phosphate-chloride exchange was measured at the higher phosphate concentrations: at 189 mM phosphate, a value close to the saturating concentration (K_s = 80 mM, Ho and Guidotti, 1974), the average inhibition was 35% (5 determinations), compared to an average inhibition of 47% at 8 mM phosphate. A double-reciprocal plot of the data (1/Flux *vs.* 1/phosphate concentration) seems to indicate chiefly a change in V_{max} (to 0.51x), and a minimal change in K_m, after ETC treatment.

2. Possible Mechanisms

The possible pathways by which a carbodiimide could react with protein carboxyl residues have been well summarized elswewhere (Hoare and Koshland, 1967; Hoare and Koshland, 1972; Pedemonte and Kaplan, 1986), and are shown in Figure IV.1. The possibility that the irreversible inhibition of anion exchange is caused by a mechanism that would result in stable incorporation of the carbodiimide into the protein, such as base-catalyzed rearrangement of an *O*-acylisourea adduct (Figure IV.1(d)), has been ruled out by the lack of incorporation of [^{14}C]ETC into Band 3 (Craik and Reithmeier, 1985). Another possibility that has been suggested (Craik and Reithmeier, 1985) is that a crosslink between the activated carboxyl and a nucleophilic group in a protein may be formed (Figure IV.1(c)). We have no evidence that this occurs, and have observed no crosslinked products among the 4 kDa pepsin-generated fragments (Ramjeesingh, Gaarn and Rothstein, 1984), or larger proteolytic fragments, of Band 3 (data not shown). An alternate reaction that could lead to irreversible inhibition is that of an exogenous ETC-activated carboxyl

Figure IV.1. Reaction pathways for the modification of carboxyl residues with a carbodiimide. The examples shown in the upper part of the figure are for an example of carbodimide reaction with an endogenous carboxyl group in a protein. The initial product of reaction between a carboxylate and a carbodiimide is an unstable *O*-acylisourea, sometimes referred to as an "activated" carboxyl group, which can undergo several possible reactions. It can rearrange to the stable *N*-acylurea (d) by an intramolecular acyl transfer, resulting in incorporation of the carbodiimide into the protein. It is also subject to attack by a nucleophile, which would yield an acyl-nucleophile product and release the carbodiimide as the corresponding urea. One special case is that of water acting as a nucleophile (a), which regenerates the carboxylate. The *O*-acylisourea can also react with an exogenous nucleophile, denoted as HX (b), resulting in covalent labeling of the protein by the nucleophile, again releasing the carbodiimide as a urea. In the case of an endogenous nucleophile (c), here shown as an amino group ($R-NH_2$), this would result in the formation of an intramolecular amide crosslink between the two protein residues involved. The scheme shown in the lower part of the figure (e) represents an alternate reaction pathway in which the reaction of the carbodiimide is with an exogenous, non-protein carboxyl group. The specific case of reaction with a carboxylate of citrate is shown since this reaction is proposed in the text. The reaction of the carbodiimide-activated citrate with a nucleophilic amino group in a protein is also shown, and would result in incorporation of citrate into the protein, and release of the carbodiimide as the urea.


(a)
R-N-C-N-R'
H2O
(b)
C-X
NH2
HX
exogenous
nucleophile
C-OH
NH2
R-N=C=N-R'
H+
R
NH
O-C
N+
R'
endogenous
carboxyl
O-acylisourea
internal
rearrangement
R-NH2
endogenous
nucleophile
NHR'
N
R
NH2
C
NH
(d)
(c)
exogenous
carboxyl
C-OH
CH2
HO-C-COO-
CH2
COO-
R
N
C
N
R'
(CIT)-C
C-NH-R
+NH
R'
NH
C-OH
(e)
CH2
HO-C-COO-
CH2
COO-
NH2
C-OH

reacting with an essential lysine side chain (or other nucleophile) in Band 3 (Figure IV.1(e)).

3. Involvement of Citrate in the Inhibition by ETC

A potentially reactive exogenous carboxyl present during treatment with ETC is the buffering ion, citrate. To test whether citrate was involved in the inhibition of anion exchange by ETC, the dependence of the level of inhibition produced on the citrate concentration in the reaction medium was measured. Treatments were performed using the same concentration of ETC (4.5 mM) in a series of citrate-buffered sucrose solutions of increasing citrate concentration. For the measurement at zero citrate concentration, modification was carried out in citrate-free media, substituting PIPES, a non-carboxyl buffer that contains no groups activatable by ETC, no potential nucleophilic groups, and is not expected to be transported by the anion transporter. When treatment of cells with 4.5 mM ETC (for 5 min at 37 °C) was carried out in PIPES-buffered sucrose (30 mM PIPES, pH 6.3, 220 mM sucrose), and transport was measured in the same buffer, the resulting level of inhibition was 12% (average of 4 determinations). With increasing citrate concentrations the resulting inhibition increased from an average of 13% in 1 mM citrate to an average of 60% in 5 mM citrate (Figure IV.2). Little additional increase in inhibition was observed with citrate concentrations up to 28.5 mM. At concentrations of citrate above 4.5 mM the concentration of ETC available to generate the activated complex might become limiting. However, attempts to increase the inhibition by using higher concentrations of ETC (up to 30 mM) rarely produced higher than 60% inhibition. The level of 60% inhibition of anion transport is in very close agreement with the values obtained by Craik and Reithmeier in 28.5 mM citrate-buffered sucrose. Thus, it appears that the production of inhibition of anion transport (above 12%) by ETC in citrate-containing media is dependent on the presence of citrate.

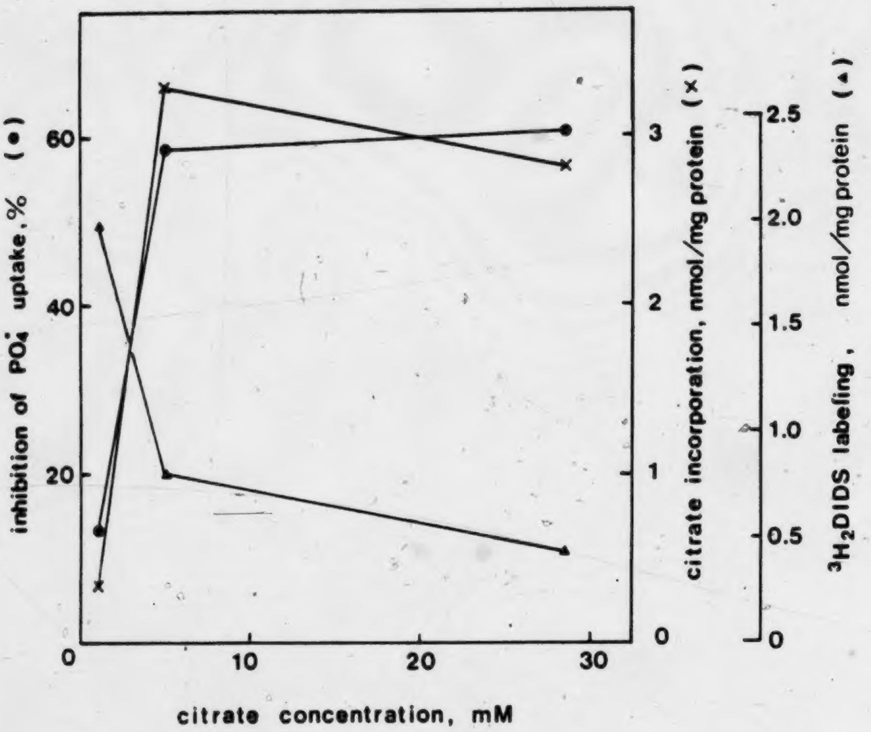


Figure IV.2. Variation of (●) inhibition of phosphate uptake, (x) $[^{14}C]$citrate incorporation, and (▲) $^3H_2$DIDS labeling with the citrate concentration present during ETC treatment. Treatment of cells with 4.5 mM ETC was done as described in Methods except in a series of citrate-buffered sucrose solutions of citrate concentration varying from 1 to 28.5 mM. The calculated osmolarity was maintained by addition of extra sucrose in these solutions. After ETC treatment, the cells were washed, and transport was measured in 28.5 mM citrate, 205.3 mM sucrose. The points for inhibition of phosphate uptake for each concentration represent an average of from 2 to 7 determinations. $[^{14}C]$citrate incorporation and $^3H_2$DIDS labeling were measured as described in Figure IV.3 and in Methods, but on cells that had been treated with ETC in citrate-buffered sucrose containing 1, 5, or 28.5 mM citrate. For citrate incorporation each point represents a single determination. For $^3H_2$DIDS labeling the points represent 3, 2 and 7 determinations, respectively, for 1, 5 and 28.5 mM citrate. Note: The level of $^3H_2$DIDS labeling of untreated cells in this experiment was 2.13 nmol/mg membrane protein. In the case of $^3H_2$DIDS labeling of cells treated with ETC in 5 mM citrate the inhibiton of transport was only 48% rather than the average of 57%.

4. Incorporation of Citrate into Band 3

If the dependence of transport inhibition on the presence of citrate is due to the reactions suggested above, in which ETC activates citrate and the complex then reacts with a nucleophile in Band 3 protein, then incorporation of citrate into Band 3 protein would be predicted. Inclusion of [^{14}C]citrate in the reaction medium during treatment of erythrocytes with ETC resulted in incorporation of [^{14}C]citrate into Band 3. The incorporation was dependent on the concentration of citrate present during treatment (Figure IV.2). At a concentration of 1 mM citrate, incorporation of 0.33 nmol citrate/mg total membrane protein was achieved, at 5 mM citrate, incorporation was 3.3 nmol/mg, and at 28.5 mM citrate the value was 2.8 nmol/mg. For the treatment in 5 mM citrate the fraction of the total radioactivity in the membranes that is associated with Band 3 was $\cong$0.52 (determined from a gel identical to lane 1 in Figure IV.3). Some radioactivity (9-15% of total) was associated with a band that migrated just below Band 3 (Figure IV.3B, lane 1), which most likely corresponds to glycophorin[3]. Correction of the amount of citrate incorporated into membranes for the fraction in Band 3 gives values of 0.17, 1.72 and 1.46 nmol/mg membrane protein, for 1, 5 and 28.5 mM citrate, respectively. Assuming Band 3 represents 25% of the membrane protein, the stoichiometry of citrate labeling is 0.07, 0.7, and 0.6 moles citrate/mole Band 3, respectively. Apparently, even at the highest levels, incorporation of [^{14}C]citrate does not represent modification of all molecules of Band 3.

The effect of occupation of the stilbenedisulfonate binding site on the labeling produced by ETC plus 5 mM [^{14}C]citrate is shown in Figure IV.4. The presence of 50 μM DNDS during the ETC plus [^{14}C]citrate treatment reduced the [^{14}C]citrate labeling of Band 3 to 24% of the labeling with no DNDS. Transport is inhibited by only 5% when cells were treated with ETC plus citrate in the presence of similar concentrations of DNDS

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[3] The position is that expected for glycophorin, as confirmed by Stains-All staining of an identical gel. In addition, chymotrypsin treatment of cells is known to remove an extracellular fragment of glycophorin, leaving a membrane-bound portion that migrates at about 68 kDa (Dzandu *et al.*, 1985). Our results indicate this treatment removed the label in the region of glycophorin (Figure IV.2B, lane 2).

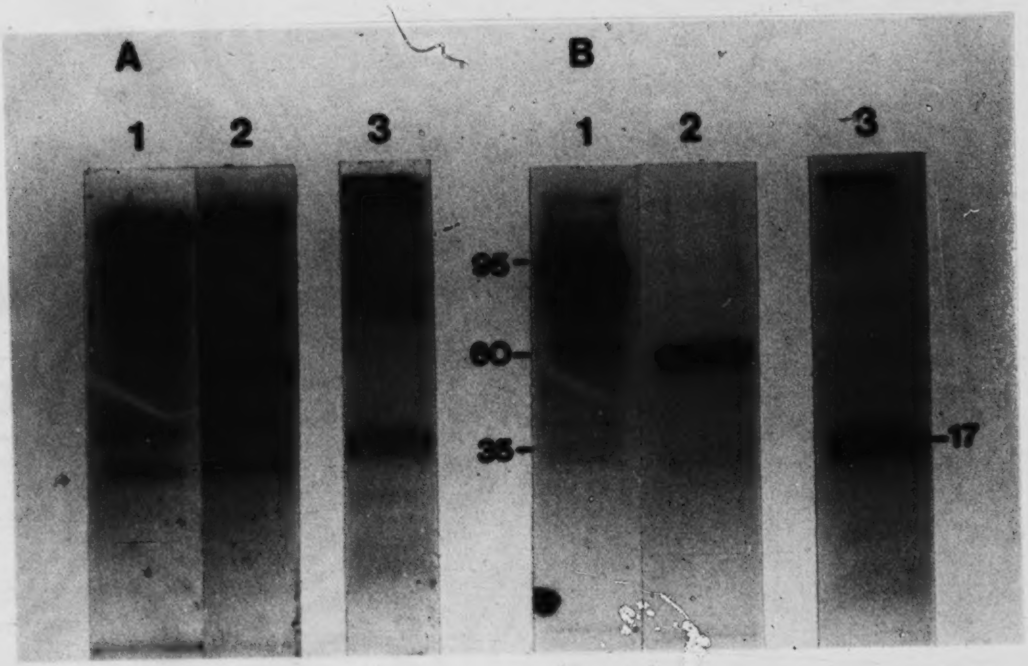


Figure IV.3. Incorporation of [^{14}C]citrate into erythrocyte membrane proteins during treatment with ETC. Erythrocytes were treated with ETC (4.5 mM) in 5 mM [1,5-^{14}C]citrate, 285 mM sucrose, the cells were washed, and ghost membranes were prepared (in sample 2, after proteolysis). A. SDS polyacrylamide gel electrophoresis of ghost membranes from the ETC plus [^{14}C]citrate treatment. Samples are: 1, no proteolysis; 2, α-chymotrypsin treated cells; 3, α-chymotrypsin treated ghosts, stripped with 2 mM EDTA, pH 12. Lanes 1 and 2 contain 10% acrylamide, while lane 3 is from a separate gel containing 15% acrylamide. B. Fluorogram of the gel lanes shown in A. The numbered bands indicated represent the fragments mentioned in the text: 95 kDa intact Band 3, the 60- and 35- kDa fragments in chymotrypsin treated cells, and the 17 kDa fragment in chymotrypsin treated ghosts. The band in lane 3 (designated 17 kDa) co-migrated with a similar band from $^3H_2$DIDS labeled cells (not shown), which is known to represent the 17 kDa $^3H_2$DIDS-binding chymotryptic fragment.

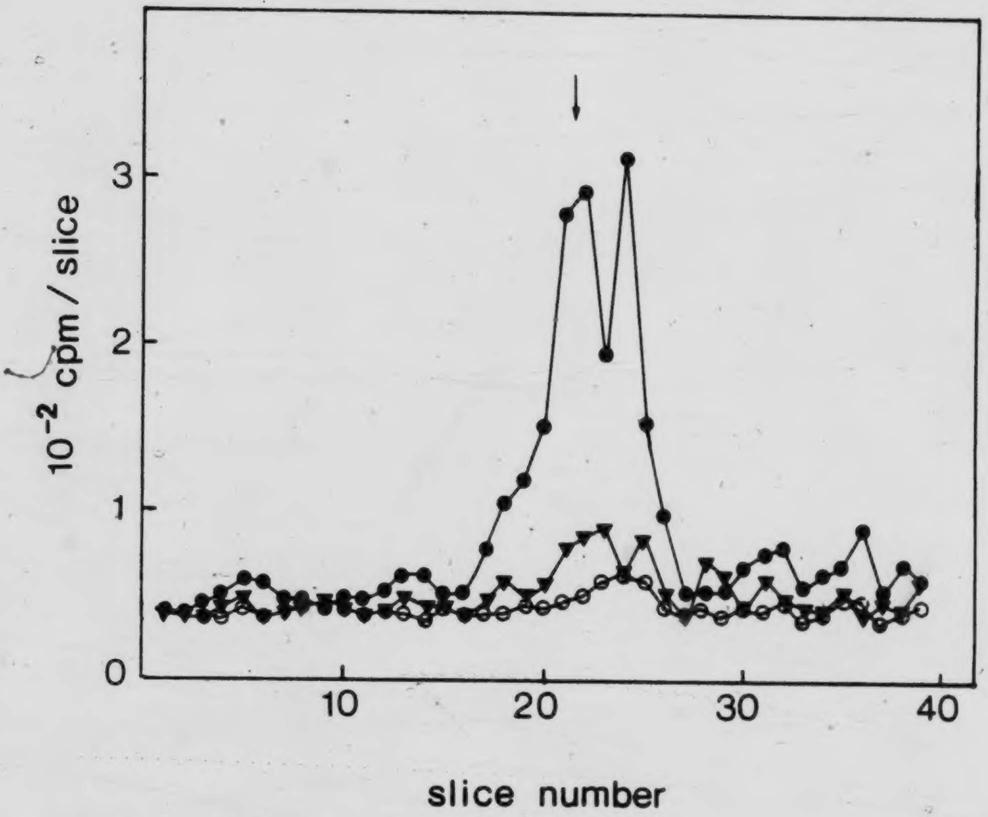


Figure IV.4. Effect of stilbenedisulfonates on the incorporation of [^{14}C]citrate into erythrocyte membrane proteins. Erythrocytes were treated with ETC in 5 mM [^{14}C]citrate, 285 mM sucrose, as in Figure IV.3, in three batches, either with no other treatment (●), in the presence of 50 µM DNDS (▼), or after pre-treatment with 10 µM DIDS at pH 7.4 (O). After washes and membrane isolation, equal amounts of membrane protein from each preparation were run on an SDS polyacrylamide gel containing 8% acrylamide. The arrow indicates the position of the stained Band 3.

(Craik and Reithmeier, 1985), whereas in cells treated with ETC plus citrate without DNDS, transport was inhibited by 57%. This shows that DNDS protects against both the irreversible inhibition of anion transport and the citrate labeling of Band 3 produced by treatment with ETC in citrate. Pre-treatment of cells with 10 μM DIDS, which should produce complete reaction of DIDS with Band 3, reduced the labeling of Band 3 by citrate to 5% that of the control.

The protection from citrate labeling by DNDS is not totally specific for Band 3. Glycophorin is also substantially protected from [^{14}C]citrate labeling by the presence of DNDS or DIDS. The protection of glycophorin is unexpected, and there are a number of possible explanations: glycophorin could be labeled on an internal site made accessible by Band 3-mediated entry of ETC-activated citrate, which would be inhibited by stilbenedisulfonates; or, more likely, the site may be external and is in some way protected by stilbenedisulfonate binding to Band 3, or to glycophorin.

To determine the relative location of the citrate in Band 3 protein, proteolytic fragmentation was performed. Fragmentation of Band 3 by chymotrypsin treatment of the cells that had been treated in 5 mM [^{14}C]citrate indicated that most of the incorporated citrate is in the NCh-60 amino-terminal chymotryptic fragment. Of the radioactivity in NCh-60 + ChC-35, that in NCh-60 accounted for about 90% (Figure IV.3B, lane 2). Upon further fragmentation of unsealed ghosts with chymotrypsin, a procedure that generates a transmembrane subfragment of 17 kDa from NCh-60, most of the radiolabel remained associated with the 17 kDa fragment (Figure IV.3B, lane 3, from a separate gel).

The citrate concentration-dependent, stilbenedisulfonate-protectable, incorporation of [^{14}C]citrate into a small region of the protein, with a maximum stoichiometry of 0.7 mol/mol provides a direct demonstration that citrate modification of Band 3 is involved in the inhibition of anion exchange by ETC. Although the data above strongly suggest that citrate modification of Band 3 is involved in the production of inhibition of transport, and limit the modification to the 17 kDa chymotryptic fragment , they do not locate a specific site of citrate modification.

5. Attempts at Localizing the Citrate-Labeled Residue(s)

In order to further localize the citrate labeled residues we attempted to generate smaller labeled peptides. It was reasoned that since the citrate modification site is likely to be accessible from the outside of cells, as the DIDS-reactive site is, that the citrate-labeled peptide might be removed by vigorous treatment of membranes with proteases. Chymotrypsin treatment of ghosts, followed by stripping (2 mM EDTA, pH 12), released up to 30% of the citrate labeled material from ghost membranes. The nature of the labeled material that was released was not further studied. The majority of the label remained associated with the membranes, in the 17 kDa fragment as shown above.

The approach used to localize the label within the 17 kDa fragment was to first separate the fragment by SDS polyacrylamide gel electrophoresis and then treat the isolated fragment with a protease. We utilized the murine sequence information to determine where predicted cleavages with certain proteolytic enzymes might be located. Because of the limited specificity of cleavage, and location of predicted cleavage sites, *Staphylococus aureus* V8-protease was used. Based on the murine sequence, using conditions resulting in cleavage only at Glu residues, 9 internal cleavage sites were predicted, producing fragments containing (listed in order from N-terminal to C-terminal): 15, 61, 10, 33/34, 7, 5, 23, 14, 13, and 18 amino acid residues. This would produce peptides ranging in molecular weight from about 8.4 kDa to about 1 kDa. The 2nd, 3rd and 4th of the potential cleavage sites are confirmed in the human sequence. Two lysine residues that are candidates for the DIDS and citrate modification (Lys *a*) would be in the most C-terminal of these peptides. This peptide contains 18 residues, and the lysine residues are located within 3 and 6 residues from the N-terminus of this peptide, making the peptide a candidate for automated sequencing methods.

As a preliminary experiment, cleavage of the 17 kDa fragment of Band 3 that had been treated with non-radioactive citrate was performed. Slices of the 17 kDa band from a gel were placed in an SDS-urea gel, V8 protease was added in increasing amounts from 1/100 to 1/2 mg protease per mg protein fragment (Cleveland *et al.*, 1977). The cleavage pattern visualized by silver-staining indicated the production of a fragment of defined size of about 8 kDa, and some smaller faintly-staining material. Because the largest of the fragments predicted was expected to be about 8 kDa, and a fragment of this size appears to have been generated, this result was considered promising. Additional experiments following this approach, but using separation techniques more suited to resolving peptides of the predicted sizes, are suggested.

6. Loss of $^3H_2$DIDS Covalent Labeling in ETC-Treated Cells.

To further investigate the effects of citrate modification on Band 3, the effect on the interaction with stilbenedisulfonate inhibitors was studied. To result in incorporation of [^{14}C]citrate into the 17 kDa chymotryptic fragment of Band 3, an ETC-activated citrate could theoretically react with any nucleophilic group in this fragment. The DIDS-reactive lysine residue, Lys *a*, is a notable candidate. Lys *a* (defined by its reactivity with the isothiocyanate derivatives of stilbenedisulfonates) is localized in the 17 kDa chymotryptic fragment (Ramjeesingh, Gaarn and Rothstein, 1980; Mawby and Findlay, 1982), and corresponds to either lysine 558 or 561 in the murine Band 3 sequence (Kopito and Lodish, 1985a). The reactivity of this residue to H_2DIDS would provide an indicator of whether it is affected by the citrate modification. Measurement of the $^3H_2$DIDS labeling of cells at pH 7.0, conditions where one end reacts with Lys *a*, indicated that the citrate modification was accompanied by a reduction in the $^3H_2$DIDS labeling of Band 3. $^3H_2$DIDS treatment of cells that had not been treated with ETC resulted in incorporation of 1.85 (± 0.35) nmol $^3H_2$DIDS/mg membrane protein (6 determinations)(see Table IV.1).

TABLE IV.1 COVALENT LABELING OF UNTREATED AND ETC-TREATED CELLS WITH [^{3}H]H$_2$DIDS

	inhibition of phosphate influx (%) @	nmol ^{3}H$_2$DIDS / mg ghost protein		sites lost	
		untreated	ETC-treated	(nmol / mg ghost protein)	% sites lost
	55	1.38	0.45	0.93	67
	58	1.31	0.21	1.10	84
	58 *	2.00	0.39	1.61	80
	59 §	2.04	0.65	1.39	68
	58	2.10	0.50	1.60	76
	67	2.17	0.52	1.65	76
AVG.	59 (±4)	1.85 (±0.35)	0.45 (±0.15)	1.38 (±0.30)	75.(±7)

[^{3}H]H$_2$DIDS labeling was performed in pairs on ETC-treated and untreated cells, for which the inhibition of phosphate influx had been determined. Standard conditions for ETC treatment were used (described in Methods). Treatment with [^{3}H]H$_2$DIDS was performed on a 25% suspension of cells in PBS, pH 7.4, with 24-50 µM ^{3}H$_2$DIDS, for 90 minutes at 37 °C.

@ Inhibition after ETC treatment, compared to transport in untreated cells.

* Transport measurement after ^{3}H$_2$DIDS treatment showed > 95% inhibition for both untreated and ETC-treated cells.

§ A portion of the non-ETC-treated sample of this set , which had been previously treated with chymotrypsin, was exposed to pH 9.5 after washing away the unreacted ^{3}H$_2$DIDS. A polyacrylamide gel of this sample indicated close to quantitative regeneration of a 95 kDa species form the 60 kDa fragment.

The $^3H_2$DIDS labeling in membranes from cells that had been treated with ETC in 28.5 mM citrate-buffered sucrose (4.5 mM ETC, producing inhibition of transport of 59 (±4) % for 6 determinations) was 0.45 (±0.15) nmol/mg membrane protein. This represents a loss of $^3H_2$DIDS labeling of 1.4 nmol/mg membrane protein, or a 75% loss of sites. Again, as with the incorporation of citrate, the modification responsible for the observed loss of $^3H_2$DIDS labeling does not occur in all molecules of Band 3, but the loss of $^3H_2$DIDS reaction is prevented to the same extent that citrate modification of Band 3 occurs.

In addition, the $^3H_2$DIDS treatment of ETC plus citrate-modified cells was sufficient to increase the level of inhibition of anion exchange from 60% to greater than 95% (data not shown). This sensitivity of the residual transport in citrate-modified cells demonstrates that even the reduced level of covalent reaction with $^3H_2$DIDS in these cells is enough to fully inhibit anion transport. This suggests the possibilities that either the citrate-modified Band 3 is fully inactive, or any residual transport by the modified transporters is able to be inhibited by $^3H_2$DIDS covalently bound to 25% of the Band 3 molecules (see Discussion).

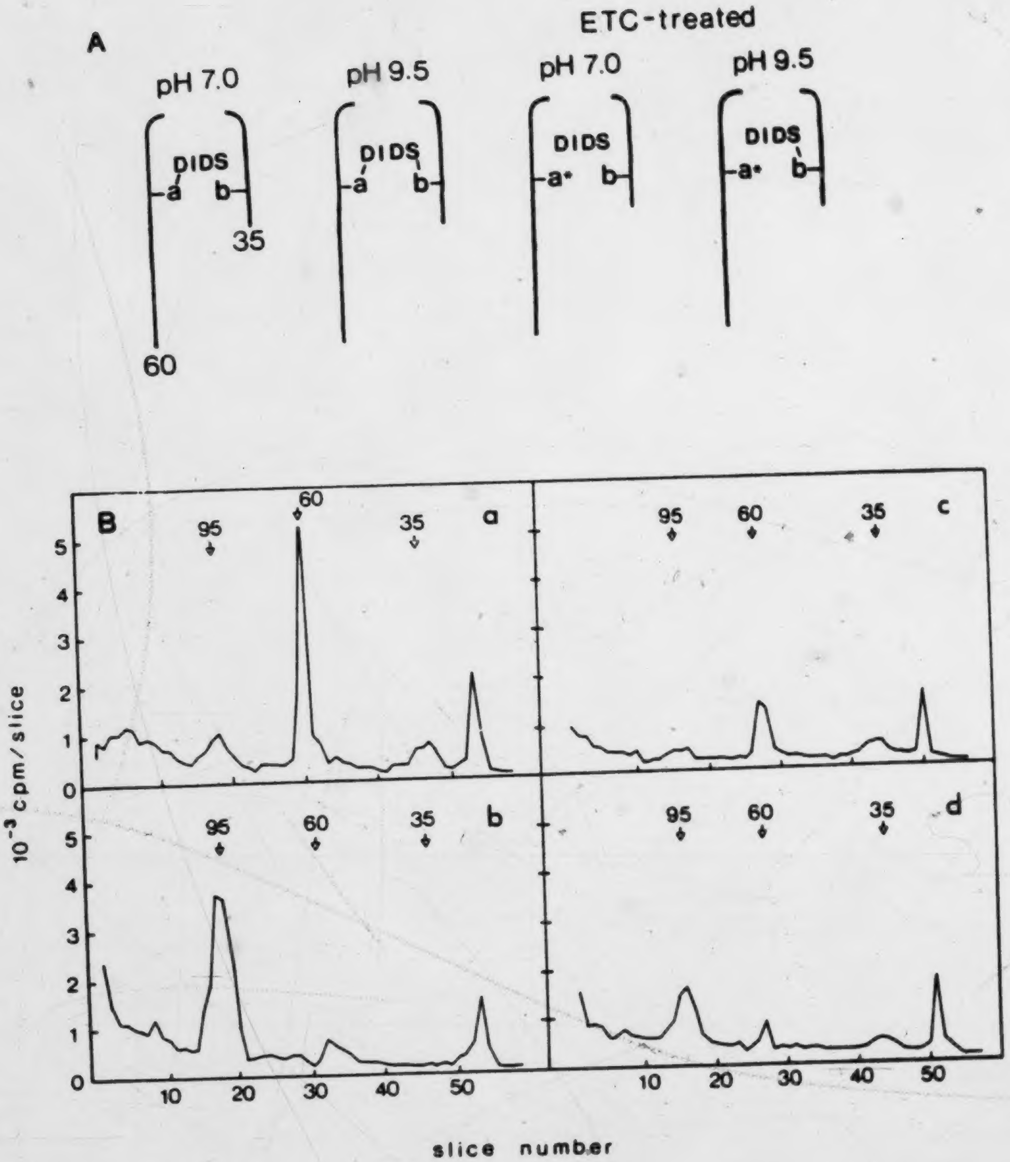
The level of $^3H_2$DIDS labeling of Band 3 was inversely dependent on the citrate concentration in the ETC reaction medium (Figure IV.2). At concentrations of citrate producing sub-maximal and maximal inhibition (1 mM and 28.5 mM, respectively) the $^3H_2$DIDS labeling stoichiometries represent a loss of $^3H_2$DIDS labeling of 0.14 nmol/mg and 1.4 nmol/mg membrane protein, respectively. As already mentioned, with increasing citrate concentrations both the inhibition of anion exchange and the incorporation of [^{14}C]citrate into Band 3 increase and reach a maximum. In addition, the H_2DIDS labeling of Band 3 decreases with increasing citrate concentration, with a similar concentration dependence as the other effects.

7. Bifunctional Reaction of $^3H_2DIDS$

The $^3H_2DIDS$ labeling results were confirmed and extended in a similar experiment in which cells were first treated with chymotrypsin before treatment with $^3H_2DIDS$. This allows the labeling of the chymotryptic fragments of Band 3, NCh-60 and ChC-35, to be determined separately, and also allows measurement of the crosslinking reaction of $^3H_2DIDS$ between the two fragments. To help understand these results, we present a cartoon illustrating the monofunctional and bifunctional $^3H_2DIDS$ reactions (Figure IV.5.A). This cartoon also presents a hypothetical view of the effect of the ETC treatment on the $^3H_2DIDS$ reaction, in which a fraction (of about 75%) of the Band 3 molecules posess a blocked lysine *a*. ETC plus citrate treatment of the cells reduced the subsequent $^3H_2DIDS$ labeling of the NCh-60 fragment by about 80% (Figure IV.5.B, panel c), similar to the reduction observed in intact Band 3 (described above). Furthermore, the quantitation shows that, although the labeling of ChC-35 by $^3H_2DIDS$ at pH 7.0 is also somewhat reduced in ETC plus citrate treated samples, the loss of labeling is preferentially from NCh-60 rather than ChC-35 (with the implication that the impairment is in the reaction with Lys *a*, not with Lys *b*). In addition, most of the chymotrypsin-cleaved Band 3 molecules that are labeled with $^3H_2DIDS$ in ETC-treated cells are able to regenerate a 95 kDa species (panel d). These molecules must not have been modified at Lys *a* or Lys *b* by the ETC-citrate treatment. Since the ability of $^3H_2DIDS$ to crosslink the unmodified NCh-60 fragment and the ChC-35 fragment is impaired only slightly more than the overall reduction in covalent labeling, substantial modification of Lys *b* by carbodiimide-activated citrate is unlikely. This is in accord with the result of only low levels of incorporation of [^{14}C]citrate into the ChC-35 fragment.

Another point that should be emphasized is that ETC plus citrate treatment of cells does not promote monofunctional reaction of $^3H_2DIDS$ with Lys *b* even at pH 9.5. There was no quantitative labeling of ChC-35 at pH 9.5 by $^3H_2DIDS$ in ETC plus citrate-treated cells (Figure IV.5.B panel d). The results above provide evidence against substantial

Figure IV.5. Effect of modification with ETC plus citrate on the $^3H_2$DIDS labeling and crosslinking of the 60 kDa and 35 kDa chymotryptic fragments of Band 3. Aliquots of ETC plus citrate treated cells and of untreated cells were digested with α-chymotrypsin and each batch of cells was divided into two portions which were then treated with 30 µM $^3H_2$DIDS at either pH 7.0 or pH 9.5. **A**, Cartoon of the situations expected in each of the four cases. The letters represent Lys *a* (a) and Lys *b* (b) in the 60 kDa and 35 kDa fragments, respectively. The pair on the left illustrate the well characterized monovalent and divalent reaction of $^3H_2$DIDS with the Band 3 residues under the two conditions (with $^3H_2$DIDS represented by DIDS). The cartoons for the ETC treated cases represent only that fraction of the protein which has been modified, with the modification assumed to be of Lys *a* (designated as an a*). Because of this assumption, the pair of cartoon figures on the right-hand side are only to be regarded as a hypothetical view presented to aid in understanding the results of the $^3H_2$DIDS labeling shown in B. **B**, Profiles of the radioactivity in the $^3H_2$DIDS labeled membranes after separation by SDS polyacrylamide gel electrophoresis. Equal amounts of ghost protein from each treatment were applied to a gel containing 10% acrylamide. Small arrows indicate the position of the crosslinked, 60- and 35- kDa fragments, and the four panels represent: a. no ETC, pH 7.0; b. no ETC, pH 9.5; c. ETC-treated, pH 7.0; d. ETC-treated, pH 9.5.


A
ETC-treated
pH 7.0
pH 9.5
pH 7.0
pH 9.5
DIDS
-a b-
-a* b-
35
60
B
a
b
c
d
95
60
35
0
1
2
3
4
5
10
20
30
40
50
10^-3 cpm/slice
slice number

alteration of the inherent reactivity of the Lys *b* residue in this fragment. Reaction of H_2DIDS with Lys *a* may be necessary for subsequent reaction of the other isothiocyano group on H_2DIDS with Lys *b*, as has been suggested by one model of the crosslinking reaction (Kampmann *et al.*, 1982). Alternatively, perhaps the inhibitor binding site is altered by the citrate modification such that $^3H_2$DIDS is oriented in an unfavorable orientation relative to Lys *b*. This would be consistent with the observed alteration in noncovalent binding affinity (see below).

8. Noncovalent Binding of Stilbenedisulfonates

Measurement of noncovalent binding of stilbenedisulfonates was carried out to determine whether the loss of $^3H_2$DIDS labeling in ETC-treated cells was due to some alteration of noncovalent binding of stilbenedisulfonates, which could reduce the subsequent covalent reaction. Measurement of noncovalent binding of $^3H_2$DIDS, the same stilbenedisulfonate that was used to measure covalent labeling, was performed under conditions in which the covalent reaction of the isothiocyano groups is suppressed, while the noncovalent association still occurs (Lepke *et al.*, 1976). Analysis of the data as a Scatchard plot (Figure IV.6) showed that the affinity of noncovalent binding of $^3H_2$DIDS by ghosts from ETC-treated cells is distinctly reduced compared to untreated membranes. However, the presence of a large component of nonspecific binding also observed by Lepke *et al.* (1976) made it impossible to estimate the exact affinities or number of sites involved in the specific stilbenedisulfonate binding. Attempts at measurement of the nonspecific component, and correction for it, were not successful. In addition, the measurements often were complicated by a substantial covalent component of the binding, although cautions noted in Lepke's work were heeded.

For these reasons, the binding of another stilbenedisulfonate inhibitor, BADS, was measured, the important feature being that this analog can bind only noncovalently since it lacks the reactive isothiocyano groups of $^3H_2DIDS$[4]. Measurement of binding of BADS to Band 3 by fluorescence enhancement titration (Figure IV.7) indicated no substantial loss of maximum binding in ETC-treated membranes. The titrations showed normal hyperbolic saturation, and the double-reciprocal plots of the data (not shown), from which the binding parameters are derived, were linear. The maximum fluorescence intensity attained in a titration of BADS binding to ghost membranes from ETC-treated cells (F_{max}= 37.0) was comparable to that of untreated membranes (F_{max}= 42.5). Inasmuch as the maximum fluorescence intensity due to BADS binding per mg of ghost protein can provide a relative measure of the number of BADS binding sites, this indicates that the number of BADS binding sites is not altered significantly by ETC treatment. However, the BADS concentration required to produce half-maximal fluorescence enhancement ($C_{1/2}$) was two-fold higher in ETC-treated membranes ($C_{1/2}$= 12 μM, compared to 6 μM in untreated), indicating ETC treatment decreased the affinity of BADS binding to Band 3 twofold. A similar titration done at an excitation wavelength of 280 nm, in which case the fluorescence enhancement is due to resonance energy transfer from tryptophan residues near the BADS binding site to bound BADS (Rao *et al.*, 1979), produced similar results: a comparable number of sites for the two preparations (18.4 and 21.5 for untreated and ETC-treated, respectively), and a twofold reduction of the apparent affinity of binding ($C_{1/2}$) for ETC-treated (5.2 μM for ETC-treated cells, compared to 2.4 μM for untreated cells). In support of this result, a reduction in affinity of stilbenedisulfonate binding after ETC plus citrate modification was observed using another technique. Citrate-modified Band 3 bound to a SITS-Affi Gel resin was more readily eluted from the resin than unmodified Band 3 (Pimplikar and Reithmeier, 1988).

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[4] The fluorescence enhancement titration measurement of BADS binding (at an excitation wavelength of 280 nm) should measure more specifically than does $^3H_2DIDS$ the binding of stilbenedisulfonates to Band 3, without the problem of a high nonspecific component of the binding. However, use of this method assumes that the fluorescence intensity per Band 3-BADS complex is unaltered by the modification (for example, if the distance between the donor and acceptor is different, or the relative polarity of the binding region is different in ETC-treated, the same fluorescence yield will not be produced).

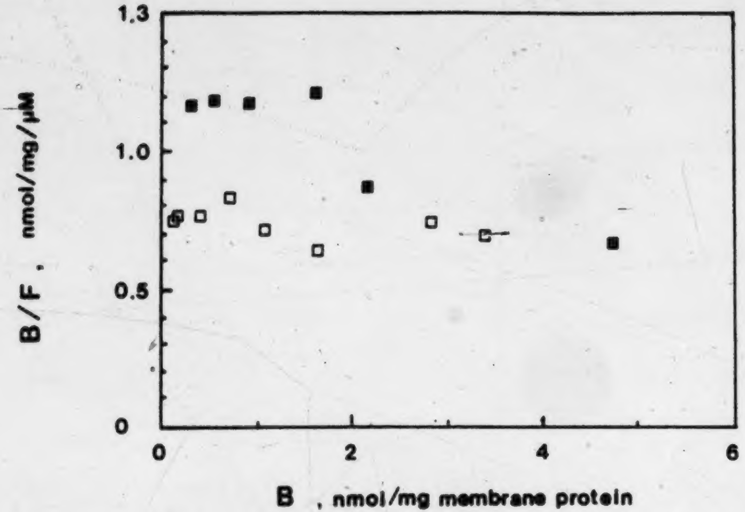


Figure IV.6. Effect of treatment with ETC in citrate media on the noncovalent binding of $^3H_2DIDS$ to Band 3. Noncovalent binding of $^3H_2DIDS$ to ghost membranes from untreated (■) and ETC plus citrate treated (□) cells was measured at 4 °C and short times to suppress covalent reaction. Ghosts in suspension, at <0.5 mg/ml protein were pelleted in an airfuge. Aliquots of suspension and supernatant were used to calculate bound (B) and free (F) $^3H_2DIDS$ concentrations, respectively.

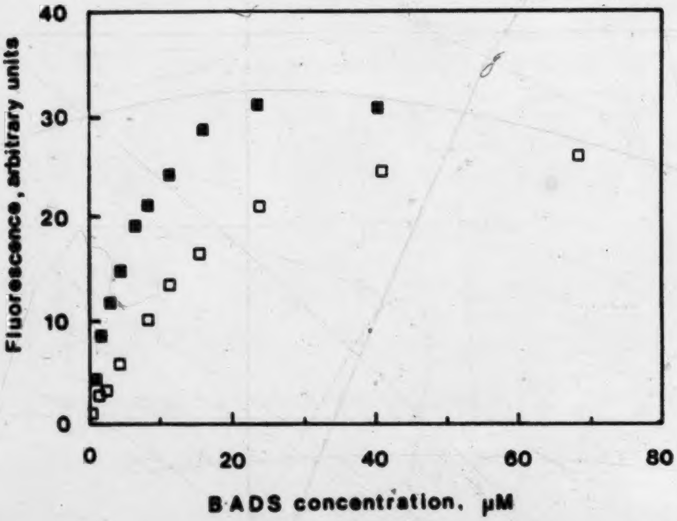


Figure IV.7. Effect of treatment with ETC in citrate media on the binding of BADS to Band 3. The binding of BADS to Band 3 in membranes from untreated (■) and ETC plus citrate treated (□) cells was measured by titration of the enhancement of BADS fluorescence that occurs upon binding to the hydrophobic stilbenedisulfonate site on Band 3. The data shown are for λ_{ex}=340 nm and λ_{em}=450 nm, and used approximately the same amount of total membrane protein.

The BADS binding curves for all titrations, including that for ETC-treated cells, produced linear double reciprocal plots, which normally would indicate a single class of homogeneous BADS binding sites. This is interesting, considering that the stoichiometry of citrate modification suggests incomplete modification of Band 3, which may be expected to give rise to two populations of BADS binding sites, due to citrate-modified and unmodified Band 3. Although the data are consistent with there being one class of binding sites, they do not exclude the possibility of the existence of two classes of binding sites with affinities so close that they cannot be distinguished.

The reduction in affinity of noncovalent binding of stilbenedisulfonates would affect the measurement of covalent labeling by $^3H_2DIDS$ only in the concentration required to achieve quantitative labeling. Since the labeling experiments were done with an excess (15 to 50 μM) of $^3H_2DIDS$, the reduction in affinity should not have contributed to the reduction in covalent labeling. Because the noncovalent binding of stilbenedisulfonates to the modified protein is not eliminated (but occurs with twofold reduced affinity), it seems likely that the reduction in $^3H_2DIDS$ covalent labeling may be due to a blocking of the groups with which the isothiocyanates react.

An estimation of the inhibitory potency of stilbenedisulfonates toward modified Band 3 would perhaps be more functionally relevant than equilibrium binding measurements. However, we are limited to assessing the apparent K_i of inhibition of the residual transport in citrate-modified cells. Anion exchange was measured in two sets of cells, ETC-treated, and untreated, in the presence of increasing concentrations of BADS (the range was 0.2 - 20 μM). The approximate K_i of inhibition of transport by BADS in untreated cells was 3 μM (the literature value is 1 μM, Lieberman and Reithmeier, 1983). The K_i of inhibition by BADS of the residual transport in ETC plus citrate-treated cells was ≅ 15 μM, a substantial increase relative to untreated cells. Because the measurement of BADS inhibitory potency reflected the binding affinity of the Band 3 molecules that are still carrying out transport, the results are most simply explained by the assumption that citrate modification of Band 3 molecules causes a change in the affinity of the unmodified Band 3. That is, the citrate modification in the stilbenedisulfonate site of one Band 3 molecule in a dimer can affect the affinity of BADS binding to the stilbene site in an unmodified protomer. If our measurements had underestimated the stoichiometry of the modification, and it was actually 1:1, then an alternate possibility would be that all the Band 3 molecules are partially inactivated. This would be in agreement with the measurements of BADS binding by fluorescence, which indicate that BADS binds with the same, reduced affinity, to all transporters in ETC-treated cells. Since it is not likely that our measurements of the stoichiometry of labeling are underestimated to the extent that would suggest 1:1 stoichiometry, the former explanation is the most probable.

D. DISCUSSION

1. Proposed Model of Inhibition

The incorporation of [^{14}C]citrate into Band 3, the loss of $^3H_2$ DIDS covalent labeling of Band 3, and inhibition of anion exchange all show a similar dependence on the citrate concentration present during ETC treatment. This correlation supports the following mode of inhibition by ETC in citrate containing media: ETC activates a carboxyl group in citrate, and the activated citrate then modifies a reactive nucleophilic residue in Band 3 (perhaps a lysine residue), thus forming a citrate-amino acid adduct that has altered transport function (see Figure IV.1(e) for a depiction of the proposed modification). In addition to sharing similar citrate concentration-dependencies, the effects of ETC treatment are also stilbenedisulfonate-protectable. The presence of 50 μM DNDS prevents to a similar extent both the inhibition of transport (Craik and Reithmeier, 1985) and the [^{14}C]citrate labeling of Band 3. Further support for the proposed mechanism comes from the close correlation between the percentage inhibition of anion exchange and the stoichiometries of both citrate incorporation and loss of $^3H_2$DIDS labeling. Incorporation of 0.7 mole citrate/mole Band 3, mostly localized in the 17 kDa Lys a-containing fragment, and loss of 75% of the covalent reaction of Band 3 with $^3H_2$DIDS, occur with treatment that results in 60% inhibition of transport.

The comparable stoichiometries of citrate incorporation, loss of $^3H_2$DIDS labeling and level of inhibition also suggests that the citrate-modified protein is completely inactive under the assay conditions, that is, that the treatment modifies only about 50% of the Band 3 molecules, but that those that are modified are inactive. That the remaining transport is due to unmodified Band 3 was indicated by the sensitivity of the residual transport to covalently bound $^3H_2$DIDS, even when only about 0.3 moles/mol Band 3 are bound. The results of anion transport measured at varying phosphate concentrations also are consistent with this view, in suggesting a reduction in the V_{max} with essentially no change in the K_m for phosphate. The alternate possibility, that the inhibition is a result of modification of all copies of Band 3, in which each is only partially inhibited, is not supported by the stoichiometries of citrate labeling or $^3H_2$DIDS labeling. However, the finding that the residual transport is inhibited by higher than normal concentrations of BADS could be consistent with either possibility.

The fact that about 50% inhibition occurs at neutral pH and further inhibition cannot easily be produced suggests that modification of one half of the Band 3 dimer with citrate precludes modification of the other half. The reduction in the K_i of BADS inhibition of the residual transport also suggests that there may be an interaction between the modification in the stilbenedisulfonate binding site on one monomer of a dimer with the binding region on the other.

Since little inhibition of anion exchange is obtained at a citrate concentration (1 mM) that produces low citrate incorporation and little loss of $^3H_2$DIDS labeling, the citrate modification of Band 3 may very well account for most of the inhibition of anion exchange by ETC in citrate media. However, the existence of a small component of the inhibition due to ETC reaction with Band 3 protein carboxyl residues cannot be entirely ruled out since exclusion of citrate from the reaction media did not result in a complete absence of inhibition. A low level of incorporation of [^{14}C]citrate into the fragment ChC-35 was also observed, but the stoichiometry (<0.1 mol/mol) was much lower than the level of inhibition of transport, suggesting it does not contribute substantially to the inhibition

The proposed mode of inhibition in citrate-containing media can accomodate the major results described by Craik and Reithmeier (Craik and Reithmeier, 1985): 1, their finding of no incorporation of [^{3}H]ETC is not surprising, since nucleophilic attack on the activated carboxyl should produce free carbodiimide urea. In fact, it is evidence for lack of incorporation of ETC into tyrosine or cysteine residues and lack of a component of reaction with Band 3 carboxyls resulting in base-catalyzed rearrangement; 2, the pH dependence of inhibition, namely, increased inhibition with higher extracellular pH, might be expected for a reaction scheme involving nucleophilic attack of a lysine residue, the deprotonated form of which acts as a nucleophile; 3, protection from inhibition by the presence of DNDS, and our companion result of protection from citrate labeling by DNDS, suggest that the modification is of a residue within the stilbenedisulfonate binding site.

We hypothesize that the site of citrate modification in Band 3 may be the particularly reactive lysine residue, Lys *a* . Lys *a* was first defined by its covalent reaction, at neutral pH, with the isothiocyanate derivatives of stilbenedisulfonates, most notably H_2DIDS and DIDS (Passow, 1986). The exact residue corresponding to Lys *a* is not known, even though a DIDS-reactive peptide has been actively pursued for many years (Passow, 1986; Ramjeesingh *et al.*, 1984; Ramjeesingh *et al.*, 1980). The amino acid sequence deduced from the cDNA for murine Band 3 has two lysine residue, Lys-558 and Lys-561, in the appropriate region in the 17 kDa chymotrypsin fragment (Kopito and Lodish, 1985). Both the localization of the incorporated [^{14}C]citrate to the 17 kDa chymotrypsin fragment, and the loss of H_2DIDS reaction after ETC-treatment in citrate are consistent with the proposal that the modified residue in Band 3 is Lys *a*. It is important to recall that the evidence for a direct modification of the residue with which H_2DIDS reacts lies in the loss of covalent

$^3H_2$DIDS labeling without loss of noncovalent binding of stilbenedisulfonates. The protection from labeling of Band 3 by citrate in the presence of DNDS could then be understood by considering the demonstrated lack of accessibility of Lys *a* to chemical modification when a stilbenedisulfonate such as DNDS is bound (Rudloff, *et al.*, 1983; Passow, 1978; Jennings *et al.*, 1985). If our proposal is correct, that the modification is of Lys *a* , then the effects of this modification can potentially further define the functional role of the Lys *a* residue and its microenvironment (see below).

That such a mechanism of inhibition occurs may not be only dependent on the high reactivity of a nucleophilic residue in Band 3. We further speculate that ETC-activated citrate may be a sort of site-directed label. The divalent citrate end of the ETC-activated citrate might bind to an external anion binding site, putting it in proximity with the reactive nucleophile. It has been shown that divalent citrate is a competitive inhibitor of the binding of a 4'-nitro-2,2'-disulfonatestilbene-spin label to the substrate site of Band 3 (Kaufmann, Eberl and Schnell, 1986).

It is conceivable that the [^{14}C]citrate label in the 17 kDa fragment is in a stilbenedisulfonate-protectable nucleophilic residue other than Lys *a*, and this change would alter noncovalent stilbenedisulfonate binding enough that covalent reaction of $^3H_2$DIDS would be impaired. We consider this possibility less likely, and note that the same criteria have been met as were used previously to define modifications of Lys *a*. Regardless of which residue in Band 3 is modified by citrate, the modification is to a significant yield, is within the stilbenedisulfonate binding site, and inhibits anion exchange. Unambiguous identification of Lys *a* as the citrate-modified residue awaits isolation of the appropriate peptide. It is interesting to note that treatment of mouse red cells with ETC (cells in citrate-buffered sucrose treated with 20 mM ETC for 5 min at pH 7.0) produced a similar level of inhibition of anion transport as in human cells. The production of inhibition in mouse red cells treated with ETC in citrate-buffered sucrose is not surprising, since it is expected that the mouse protein contains a residue with the functional role and high reactivity of lysine *a*.

2. Implications for the Role of Lys *a* in Transport and Inhibitor Binding

The characteristics of ETC-activated citrate modification of Band 3 that we describe are consistent with the proposal that Lys *a* is the residue that is modified by citrate. If this proposal is correct, the effects of this modification can potentially further define the role of this lysine residue in anion exchange. Information about the functional significance of Lys *a* comes from work using several different modification procedures (each of these examples is illustrated in Figure IV.8). Lys *a* was first defined by its reaction, at neutral pH, with the isothiocyanate derivatives of stilbenedisulfonates, most notably H_2DIDS and DIDS (Passow, 1986). The inhibition of transport by H_2DIDS is produced by noncovalent binding alone, presumably by interaction of the sulfonates with positively charged residues in the transport site (Passow, 1986; Lepke *et al.*, 1976; Shami, Rothstein and Knauf, 1978). DIDS and H_2DIDS can also react covalently with Lys *a* but, because noncovalent binding alone produces inhibition, the involvement of Lys *a* in transport could not be distinguished using H_2DIDS as an inhibitor. Dinitrophenylation of Lys *a*, which produces a bulky derivative that would be uncharged at neutral pH, leads to inhibition of transport (Rudloff, Lepke and Passow, 1983; Passow, 1978). Reductive methylation of Lys *a*, , which produces a minor steric perturbation and unaltered charge on the lysine, does not affect chloride-chloride exchange (Jennings, 1982). Evidence from both dinitrophenylation and reductive methylation experiments supports that Lys *a* is not required for the function of noncovalent $^3H_2$DIDS binding (Passow, 1978; Jennings, 1982; Jennings and Nicknish, 1984; Passow *et al.*, 1980; Schnell *et al.*, 1983). Pyridoxal phosphate (PLP) also reacts with lysine residues in Band 3 and inhibits anion exchange, however, the modification occurring in the 17 kDa fragment is not prevented by stilbenedisulfonates (Nanri *et al*, 1983). This does not agree with the definition of Lys *a*, so we conclude that the modification in the 17 kDa fragment is likely of some other residue, and it is not shown in Figure IV.8. The intramolecular crosslink formed by BSSS (bis-sulfosuccinimidyl suberate), between Lys *a* and (probably) Lys *b*, shifts the extracellular pH dependence of

Figure IV.8 Modifications of lysine *a*. The schematic depicts the lysine residues in Band 3 called Lys *a*, in the fragment CH-60, and Lys *b*, in the fragment CH-35. The depiction also shows two arginine residues near these lysine residues, the ones presumed to interact with the sulfonate groups on stilbenedisulfonates. We presume a fixed orientation of the two lysine and two arginine residues, as defined by the structure of the specific inhibitor H_2DIDS, but the planar representation is not meant to imply any further structural features. The predicted charges present at pH 7 are also indicated. The modifications are:

Top left, bifunctional reaction of dihydro-DIDS with Lys *a* and Lys *b* (also representative of mono-reacted Lys *a*), in which the sulfonates are effectively neutralized by interaction with the arginine residues;

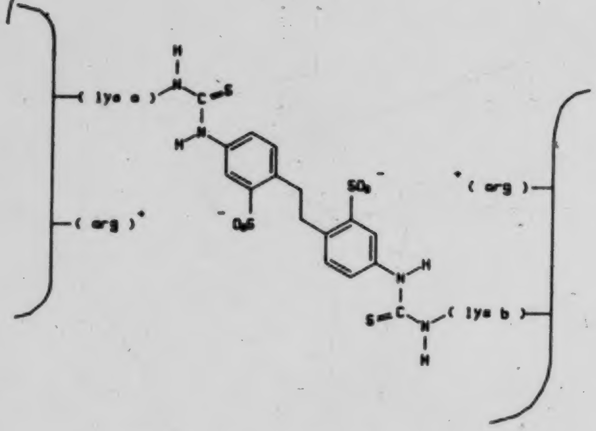
Top right, crosslinking of Lys *a* and Lys *b* by bis-sulfosuccinimidyl suberate (BSSS). This crosslink is several angstroms shorter than that with H_2DIDS, and the reacted lysine residues are thought to be in the unprotonated form at pH 7 - 8;

Bottom left, reductive methylation of Lys *a* with formaldehyde plus $NaBH_4$, in which R would be either H (for mono-methylated) or CH_3 (for di-methylated). The pK_a is only slightly affected, so the adduct would still be positively charged at pH 7;

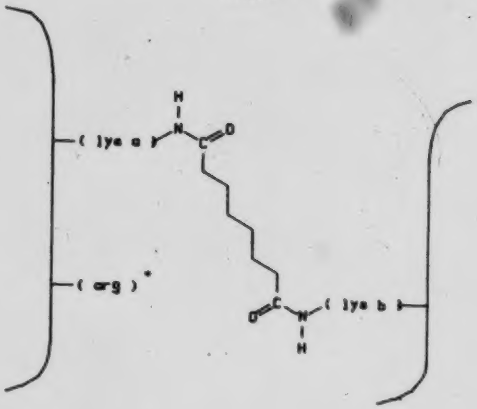
Bottom center, dinitrophenylation of Lys *a*, in which case the adduct should be uncharged at neutral or even moderately acidic pHs;

Bottom right, citrate modification of Lys *a*, as proposed herein for the inhibitory modification resulting from treatment of erythrocytes with ETC in citrate-buffered sucrose. Reaction with one of the end (highest pK_a) carboxylates is depicted, and the other two would be negatively charged at pH 7.

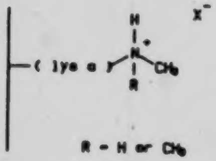
DIHYDRO-DIDS


(lys a)
(arg)+
SO3-
-O3S
+(arg)
(lys b)

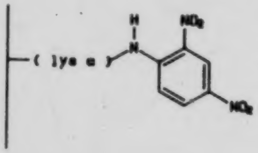
BSSS


(lys a)
(arg)+
(lys b)

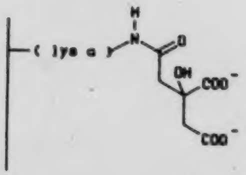
REDUCTIVE METHYLATION


(lys a)
X-
CH3
R = H or CH3

DINITROPHENYLATION
(1-F-2, 4-dinitrobenzene)


(lys a)
NO2
NO2

CITRATE


(lys a)
OH
COO-
COO-

transport down by approximately 5 pH units (Jennings *et al.*, 1985), and would be expected to block binding of stilbenedisulfonates. Although it is not clear which effects of BSSS are attributable to Lys *a*, and which to Lys *b*, the authors point out that one of the lysines provides a positive charge that has a necessary role in anion exchange. The information regarding the functional significance of this lysine residue that is provided by previous modifications of Lys *a* can be summarized as follows: Lys *a* appears not to be required for noncovalent stilbenedisulfonate (H_2DIDS) binding (Passow, 1978; Jennings, 1982; Jennings and Nicknish, 1984; Passow *et al.*, 1980; Schnell *et al.*, 1983); and the role of Lys *a* in exchange is not fully clear, but either Lys *a* or Lys *b* may provide a positive charge that has a necessary role in anion exchange (Jennings *et al.*, 1985).

The proposed citrate-modified Lys *a* that we describe is clearly different from the previous modifications (see Figure IV.8). After modification, noncovalent binding of stilbenedisulfonates still occurs, but with reduced affinity. This can be rationalized without contradicting the conclusions of the earlier work by considering that the introduction of two negative charges within the stilbenedisulfonate binding region might perturb the site sufficiently to alter the noncovalent binding affinities of stilbenedisulfonates. Citrate modification also clearly inhibits phosphate-chloride exchange, clarifying the conclusion from the BSSS results (Jennings *et al.*, 1985) that the charge on Lys *a* (rather than on either Lys *a* or Lys *b*) is important in some part of the exchange function. At the very least, it indicates that the protein with two negative charges on Lys *a* cannot maintain transport function.

How does the citrate modification inhibit transport? Preliminary results from measurements of transport at varying external phosphate concentrations indicate that the modification appears to reduce the apparent V_{max} of the exchange flux, without much change in the K_m for external phosphate. The lack of effect on the K_m is consistent with the evidence that the modified residue is not involved directly in anion binding. The conversion of a lysine of charge +1 to an adduct having a charge of -2 either could have a direct effect on the function of this residue, or could exert an effect by changing the microenvironment of the residue. Further characterization is needed to adequately understand the functional consequences of the ETC-mediated citrate modification of Band 3. It would be interesting to investigate the effects of this modification on the Cl^- binding, as measured by ^{35}Cl-NMR (Falke and Chan, 1986), and determine if the modification acts as a channel-blocking or translocation inhibitor.

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V. A SECOND MECHANISM OF INHIBITION OF ANION EXCHANGE BY THE CARBODIIMIDE ETC: INHIBITION IN THE ABSENCE OF CITRATE[1]

A. INTRODUCTION

Anion exchange transport in human erythrocytes is inhibited by carboxyl-selective reagents, as summarized in Chapter IV. Treatment with the membrane-impermeant carbodiimide ETC, in particular, was shown (in Chapter IV) to produce two distinct types of inhibition, differing in their inhibition characteristics and in their mode of production. The inhibition produced by treatment in citrate-containing media was shown to be due not to protein carboxyl modification, but to carbodiimide activation of citrate, and subsequent modification of a nucleophilic residue in Band 3. When treatment with ETC is performed in non-carboxyl-containing buffers, however, excluding an exogenous carboxyl mode of inhibition, inhibition of anion transport still occurs. The inhibition produced by ETC in the absence of citrate is presumed to be due to the modification of protein carboxyl residues. It had been characterized by its reversibility -- the inhibition remains after removing the reagent, but gradually diminishes upon continued incubation (Craik and Reithmeier, 1985). The aim of this work was to examine the nature of the inhibition produced by ETC in the absence of citrate, asking the more specific questions: is it due to a reaction of ETC with Band 3 carboxylate residues? what accounts for the observed reversibility of the inhibition? and, can we rule out the involvement of buffer ions in the inhibition in this case?

The lack of incorporation of [^{14}C]ETC into Band 3 in citrate-containing media demonstrated by Craik and Reithmeier (1985) suggests that the reactions producing stable incorporation of carbodiimide are not occurring, but this does not provide positive evidence for the reaction with carboxylate residues in the absence of citrate. Potentially more convincing, but preliminary, evidence that the transport inhibition involves reaction with carboxylate residues is provided by reports that addition of nucleophiles enhanced the rate of inhibition of transport by carbodiimides (Wieth *et al.*, 1982a; Andersen *et al.*,1983) and of carbodiimide-mediated incorporation of a nucleophile into Band 3 (Bjerrum, 1983).

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[1] A portion of this chapter has been published: Werner, P.K. and Reithmeier, R.A.F. (1988) *Biochim. Biophys. Acta.* **942**, 19-32..

As presented in this chapter, we have been successful at demonstrating that the addition of certain nucleophiles during the treatment with ETC produces stable inhibition of anion transport, apparently preventing the reversal of inhibition. This result provides strong evidence for a role of carboxyl residues in anion exchange, and indicates that modification of the carboxyls with either carbodiimide or a hydrophobic nucleophile results in inhibition. Two approaches aimed at labeling and identifying the carboxyl residues in Band 3 that are involved in this inhibition are also described in this chapter. The first is to determine whether there are conditions under which radiolabeled carbodiimide is incorporated into the protein (extending the results previously obtained in citrate-containing media). The second is to add a radiolabeled nucleophile in conjunction with the carbodiimide, to promote resolution of the carbodiimide-carboxyl intermediate by reaction with, and incorporation of, the nucleophile (Hoare and Koshland, 1969; Carraway and Koshland, 1972). Both approaches were found to result in significant labeling of glycophorin, but very little or no labeling of Band 3. Thus, these approaches will likely be of limited usefulness in labeling carboxyl residues in Band 3 protein.

B. MATERIALS AND METHODS

Carrier free [^{32}P]phosphate was obtained from New England Nuclear. 1,2-ditritio-1,2-(2,2-disulfo-4,4-diisothiocyano)diphenylethane ([^{3}H]H_2DIDS, at 202 mCi/mmol) was obtained from Research Development Corporation, Hospital for Sick Children, Toronto.. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was from Pierce Chemical Company, Rockford Illinois, and the amino acid ethyl esters were from Sigma, except nitrotyrosine ethyl ester, which was synthesized from L-nitrotyrosine (Dall'Asta and Ferrario, 1962). 1-ethyl-3-[3-(trimethylammonio)propyl] carbodiimide iodide (ETC) was prepared from the free base of 1-ethyl-3-(3-dimethylamino propyl) carbodiimide by the method of Sheehan *et al.* (Sheehan, Cruickshank and Boshart, 1961), as described previously (Craik and Reithmeier, 1985). All other chemicals were reagent grade or better from standard suppliers.

1. Preparation of Erythrocytes. Outdated human red cells, kindly donated by the Canadian Red Cross, were washed four times with 10-15 volumes of 0.9% w/v NaCl at 4 °C to remove storage medium and white cells. If treatment in another medium was desired, the cells were then washed 3 times with 10 volumes of the desired buffer. In the cases where the pH of the cell suspension was adjusted, 0.1 N HCl was added to a 5% suspension of cells in 165 mM KCl to obtain a pH of about 5. The cells were then washed twice in MES-buffered KCl, pH 5.5, and resuspended in the same buffer to produce the desired pH.

2. Treatments of Cells with ETC and Nucleophiles. Treatment with ETC was done in one of several treatment buffers, including phosphate-buffered saline (PBS, of the composition 5 mM sodium phosphate, pH 7.0, 150 mM NaCl), MES-buffered KCl (5 mM MES, pH 5.5, 165 mM KCl), and PIPES-buffered sucrose (30 mM PIPES, pH 6.3, 220 mM sucrose). Cells at 50% suspension in the desired buffer were pre-incubated at 37 °C for 5 min. ETC was added, by dilution from a freshly dissolved solution in the same buffer, to the warmed suspension, continuing incubation at 37 °C for the desired time. The stated ETC concentrations were calculated by dilution, assuming the dilution is into the external volume of the suspension only. After reaction for a designated time the cells were then washed twice with ice-cold treatment media containing 0.5% fatty acid-free bovine serum albumin and three times in the buffer to be used for the next incubation or for transport measurements. The process from the end of ETC exposure to the start of the transport measurement generally took about 15 minutes.

The nucleophiles were added from stock solutions of 150 mM of the chloride salt in distilled H_2O. The appropriate volumes of nucleophile stock and treatment buffer were added to packed cells to obtain a 50% suspension of cells and a final concentration of nucleophile of approximately 50 mM, based on extracellular volume. After pre-incubation for 5 min at 37 °C, ETC was added to the suspension, and allowed to react for the desired time (2 to 120 min). The cells were then washed 3 times with ice-cold PBS containing 0.5% bovine serum albumin before washing with transport buffer.

3. [^{32}P] PBS Treatment. Erythrocytes in 0.9% NaCl were added to an equal volume of PBS containing [^{32}P]phosphate (110 nCi/nmol), then 18 mM ETC was added and the mixture incubated at 37 °C for 5 min. At this level of radioactivity, for example, if 0.1 mol P_i per mol Band 3 were incorporated, approximately 1000 dpm per 20 μg membrane protein should be detected.

4. Transport Measurements. The exchange of extracellular phosphate for intracellular chloride was measured by following the loss of [^{32}P] phosphate from the supernatant of a 50% suspension of chloride-loaded cells in 28.5 mM citrate-buffered sucrose, pH 7.4, as described previously (Ho and Guidotti, 1975; Craik and Reithmeier, 1985; Craik and Reithmeier, 1986). Inhibition of transport was estimated from the change in half time for the initial loss of radioactivity from the extracellular medium. Inhibition (%) = (1 - ($t_{1/2}$ control/ $t_{1/2}$ treated)) x 100%. Some transport measurements were performed using PIPES-buffered sucrose instead of citrate-buffered sucrose. The half-times for untreated cells were generally slightly faster in this medium than in citrate-buffered sucrose. Provided that washes in treatment buffer (as described with each treatment) were done before washes in transport buffer, the inhibition obtained was the same, regardless of the medium in which transport was measured.

5. Preparation of Erythrocyte Membranes. Following post-treatment washing, cells were lysed in ice-cold 5 mM sodium phosphate, pH 8 (Dodge, Mitchell and Hanahan, 1963), containing 0.1 mM phenylmethylsulfonyl fluoride, and washed extensively with the same buffer. Membranes were recovered by centrifugation at 55,000 rpm in a TLA 100.2 rotor (>100,000 x g).

6. Synthesis of [^{14}C]ETC. The chemistry for the synthesis was the same as for synthesis of non-radioactive ETC, as described above, but [^{14}C]methyl iodide was used. A break-seal ampoule containing 1 mCi [^{14}C]methyl iodide, at 56 mCi/mmol (purchased from Amersham), was chilled over a dry ice-acetone bath, the vial was opened, and the reagent transfered using a small volume of anhydrous diethyl ether as a carrier solvent. The solution was then mixed with a defined volume of a solution of unlabeled methyl iodide in ether such that the specific radioactivity was reduced by one half (total 35.6 μmol, or 5.06 mg). A solution of EDC base in ether, containing 17.6 μmol, was added, the mixture capped, and left to react for 18 hours. The resulting white precipitate was washed four times with dry ether, recovering the precipitate by centrifugation, and was then dried under vacuum.

7. [^{14}C]ETC Treatments of Cells. Just before starting the reactions, the [^{14}C]ETC precipitate was dissolved in 80 μL MES-buffered KCl, pH 5.5. Based on the actual counts in the dissolved reaction product, the concentration of the stock was 78.1 mM, at a specific radioactivity of 28 mCi/mmol. Aliquots of the stock were then added to 50 μL of cells that had been prepared in the appropriate buffers, and with additions (as listed in Table V.2), and incubated at 37 °C for 1 hr. The free ETC concentration was determined from the radioactivity in the treatment supernatant. The cells were washed 3 times with 0.9% NaCl containing 0.5% fatty acid-free bovine serum albumin. Ghosts were prepared in 5 mM sodium phosphate, pH 7, containing 0.1 mM PMSF, and washed until the supernatant contained <100 dpm/100 μL. The 40 - 50 μL membrane pellet of each sample was suspended in 200 μL of the same buffer, vortexed, and aliquots of the suspension were taken for scintillation counting, protein determination, and gel electrophoresis.

8. [^{14}C]PheOEt Synthesis. The synthesis procedure, by acid catalyzed esterification of radiolabeled amino acid with ethanol, essentially follows the method for synthesis of nitrotyrosine ethyl ester (Dall'Asta and Ferrario, 1962), and is described by:

$$[^{14}\text{C}]\text{Phe} \xrightarrow{\text{HCl-saturated ethanol}} [^{14}\text{C}]\text{PheOEt}$$

50 μCi of the starting product, L-[U-^{14}C]Phenylalanine, at 513 mCi / mmol (purchased from Amersham), was lyophilized in a small, Teflon-capped vial. Absolute (anhydrous) ethanol [2] was saturated with HCl gas by gently bubbling a stream of anhydrous HCl under pressure (Linde) through the ethanol, using a Monel valve attached to a pasteur pipet, with stirring, on ice, for 5 - 10 minutes. 5 mg of nonradioactive phenylalanine was dissolved in 1.5 mL of the HCl-saturated ethanol and 0.15 mL of this solution was added to the dried down [^{14}C]Phe. The vial was capped, mixed, and heated at 40 - 45 °C in a heating block for about 6 hours. The extent of reaction, as well as a pre-reaction sample, was checked by TLC on silica gel in a solvent system of n-butanol / acetone / triethanolamine / water (6:6:1:1, volume), visualizing with Ninhydrin. At this point, the reaction was 98% complete with approximately 84% recovery. The reaction mixture was dried down overnight under a water-aspirated vacuum with NaOH pellets in the desiccator. The residue was suspended in 100 μL absolute ethanol and dried down once more.

9. Treatment of Erythrocytes with ETC and [^{14}C]PheOEt. The dried [^{14}C]PheOEt from the synthesis described above (0.42 mg) was suspended in 70 μL 100 mM sodium phosphate, pH 7.0, and added to 60 μL washed, packed red cells. ETC was added to 18 - 20 mM concentration, and the suspension was incubated at 37 °C for 30 minutes. The cells were then pelleted by centrifuging for 30 sec in a microfuge. The supernatant was removed and used to treat another aliquot of cells, adding fresh ETC. After treatment the cells were washed with cold 165 mM KCl, containing 0.5% bovine serum albumin, then with the same solution without bovine serum albumin, until the radioactivity in the wash was reduced. Three different treatments of cells with [^{14}C]PheOEt were done, and the first was processed to ghosts without further treatment. The second batch was treated with α-chymotrypsin (a 25% suspension of cells in PBS was treated with 1 mg/ml enzyme for 1 hr at 37 °C), before hemolysis, and the third portion was treated with trypsin (a 50% suspension in PBS, pH 8, was treated with 200 μg trypsin/mL at 37 °C for 1 hr) after preparation of ghosts. Aliquots of a diluted suspension of the membranes from each sample were taken for scintillation counting, protein determination, and gel electrophoresis.

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[2] It is very important that the ethanol be anhydrous. If in doubt, ethanol that has accumulated some water may be dehydrated by the magnesium ethoxide method described on pp. 249-250 in "Purification of Laboratory Chemicals", 2nd Edition, D.D. Perrin, W.L. Armarigo and D.R. Perrin , eds , (1980), Pergamon Press, Ltd.

10. Covalent $^3H_2$DIDS Labeling. Solid $^3H_2$DIDS (202 mCi/mmol) was dissolved in PBS, pH 7.0, at a concentration of 240 μM, and was stored in aliquots at -20 °C. Cells washed in PBS were treated at 25% suspension with 32 - 35 μM $^3H_2$DIDS (4.95 x 10^5 dpm/nmol), for 90 min at 37 °C (Shami, Rothstein and Knauf, 1978). The cells were then washed three times with PBS containing 0.5% fatty acid free bovine serum albumin, and finally with buffer without added albumin. To measure the level of labeling, aliquots of a diluted suspension of membranes were taken for scintillation counting, protein determination, and gel electrophoresis.

11. Treatment of Cells with ETC in the Presence of DNDS. Cells at 50% suspension in PBS, pH 7.0 were pre-incubated, either with or without addition of approximately 100 μM DNDS (based on the extracellular volume), for 5 minutes at 37 °C. After addition of ETC (at either 6 or 50 mM), and incubation at 37 °C (for either 5 or 60 minutes), the cells were washed before the transport measurement. The usual post-ETC-treatment washes (3 x 12 volumes with 0.9% NaCl containing 0.5% fatty acid-free bovine serum albumin, and 3 x 12 volumes citrate-buffered sucrose, pH 7.3, all at 0 - 4 °C) were found to be inadequate for removal of DNDS -- transport was inhibited 30 % to 90% using this washing procedure after incubation with 100 μM DNDS alone. A washing procedure that resulted in very little (0 - 5%) remaining inhibition was to use the same sequence and number of washes as usual, except to use 0.9% NaCl plus bovine serum albumin at room temperature (≅ 21 °C).

12. SDS Polyacrylamide Gel Electrophoresis, Fluorography and Densitometry. SDS polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (1970). The gels were stained with either Coomassie Blue (0.1% Coomassie Blue-R in 25% isopropanol, 10% acetic acid), or with Stains-All (King and Morrison, 1976). Densitometry of stained gels was performed on a Joyce-Loebl Chromoscan 3, using a 530 nm filter for Coomassie-stained gels, and a 626 nm filter, which emphasizes the bands staining blue, for scanning gels stained with Stains-All. Any values of quantitation for the densitometry measurements are those provided by the integration by the Chromoscan. Fluorography involved pretreatment of the gel with Enlighten enhancer (New England Nuclear), drying the gel under vacuum with heating, and exposure of the dried gel at -70 °C to Kodak X-Omat X-ray film. In one case the gel was dried directly after electrophoresis, without fixing. Calculation of the distribution of radioactivity in gels was done by densitometry of a fluorogram prepared using pre-flashed film. Densitometry was done as for Coomassie-stained gels.

13. Analytical Techniques. The protein concentration was determined according to Lowry (Lowry *et al.*, 1951), in the presence of 1% SDS, using bovine serum albumin as a standard. Scintillation counting was performed in a Beckman LS7800 scintillation counter with an external standard method of quench correction.

C. RESULTS

1. Stability of Inhibition Produced Under Different Conditions -- Both Reversible and Irreversible Inhibition Can Be Produced.

ETC treatment of erythrocytes in phosphate-buffered NaCl, in the absence of citrate, was characterized as resulting in reversible inhibition of anion exchange (Craik and

Reithmeier, 1986). It is important to clarify that by reversible inhibition we mean that the inhibition produced in phosphate-buffered NaCl remained after the ETC was removed by several washes but, unlike the inhibition produced in citrate-buffered sucrose, reversed over a period of 60 minutes' incubation in PBS (see Figure V.1 ,without nucleophile). Although it was assumed that the reversal of inhibition with time was due to a relatively slow hydrolysis of the carbodiimide-carboxyl intermediate, as illustrated in Figure IV.1(a), it is also possible that some other labile reaction could be involved in inhibition. There are suggestions in the literature that phosphate can be activated by carbodiimides in aqueous media and that cautious attention should be paid to the effect of such side reactions (George and Borders, 1979). Because treatment with ETC was usually performed in phosphate buffered saline (5 mM P_i) we wanted to rule out reaction with phosphate. Treatment with ETC in [^{32}P]PBS resulted in an incorporation of ^{32}P into the isolated membranes of 0.032 nmol P_i / mg membrane protein (for both a 5 min and a 60 min incubation), and most of the radioactivity migrated with the dye front of an SDS polyacrylamide gel (not shown). Thus, it is unlikely that a mechanism of inhibition involving reaction of activated phosphate with Band 3 occurs. However, this result does not rule out a possible reaction of ETC with phosphate that could result in an effective reduction in the concentration available to react with Band 3.

The reversal of inhibition produced in PBS was found to be alarmingly inconsistent. This was especially true when the treatment involved continuous exposure to ETC, although in some instances continued exposure produced reversal of inhibition, as shown in Figure V.1, and by Craik and Reithmeier (1985). Often, the inhibition measured after 60 minutes would be only partially reduced compared to that measured after 5 minutes of treatment (for conditions as in Figure V.1). One would expect that if the reagent were not consumed in the first five minutes that further reaction could occur with continued exposure, preventing reversal of inhibition. The reversal of inhibition did occur more consistently when the ETC was present only during the first five minutes of the incubation, with the remaining 55 minute incubation in buffer alone, rather than over the entire time period.

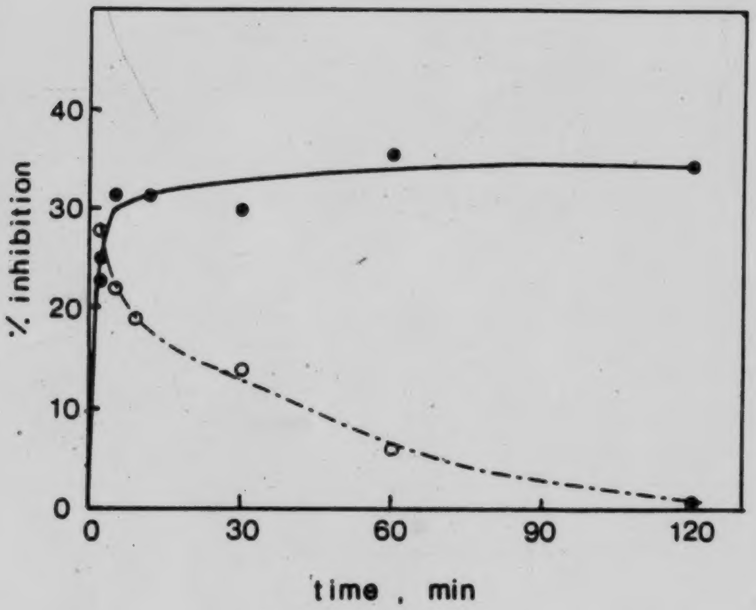

Figure V.1. Time course of inhibition of anion exchange by treatment with ETC in phosphate-buffered saline in the presence and absence of added nucleophile. Treatment of erythrocytes in PBS, at a suspension pH of 6.8, with 18 mM ETC was carried out for the designated time, either in the absence (○, ⊕) or the presence (●) of 50 mM phenylalanine ethyl ester (PheOEt). The cells were washed in PBS with 0.5% bovine serum albumin (3x), then with 28.5 mM citrate-buffered sucrose, in preparation for the transport measurement. Data points from Craik and Reithmeier (1985) are included to illustrate the usual time course in the absence of added nucleophile (○), which was confirmed (⊕) by measurement at the first and last times.

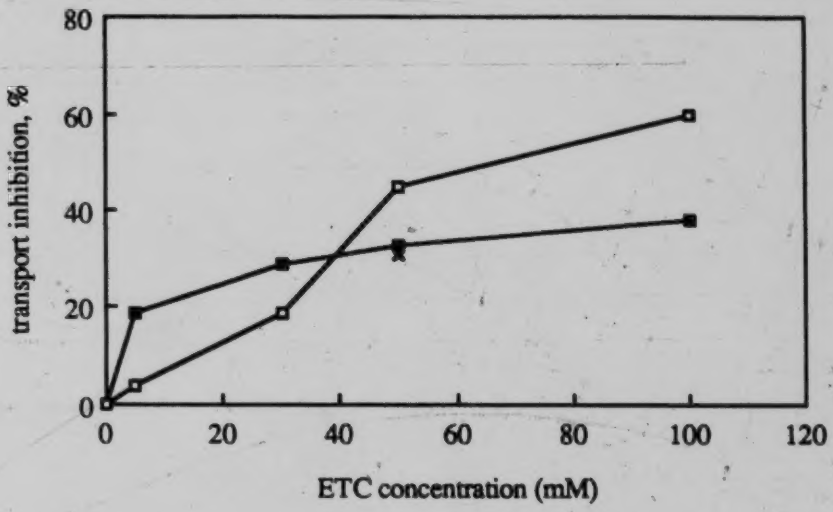

Figure V.2 Dependence of inhibition produced in phosphate-buffered NaCl on ETC concentration. Transport measurements were performed on control cells and on samples that had been treated for either 5 minutes (■), or for 60 minutes(□), with varying concentrations of ETC. One measurement (x) was also done on cells that had been treated with 50 mM ETC for 5 minutes, washed, then incubated for 55 minutes more in buffer without ETC.

The production of transport inhibition, and its reversal, appeared to be dependent on the ETC concentration. Figure V.2 shows the dependence of inhibition on the concentration of ETC during incubation with ETC for either 5 minutes, or for 60 minutes. Treatment with 5 mM ETC for 5 minutes produced a substantial inhibition, but the level of inhibition approached a maximum with increasing ETC concentrations. The dependence on ETC concentration is very different for the 60 minute treatments, with the inhibition increasing almost linearly through the entire range of concentrations used. At low concentrations of ETC (5 mM) the inhibition produced after treatment for 60 minutes was distinctly lower than after 5 minutes. This is likely due to consumption of the reagent and hydrolysis of the carbodiimide adduct over the 60 minute time period. At higher concentrations of ETC (50 and 100 mM) there was a higher level of inhibition after a 60 minute treatment when compared to a 5 minute treatment. Further, even the inhibition produced after 5 minutes' treatment with 50 mM ETC did not appear to be reversible. A sample treated with 50 mM ETC for 5 minutes, washed, then incubated an additional 55 minutes in buffer alone, showed the same level of transport inhibition as the sample treated for 5 minutes without any further incubation. In this case, the inhibition is unlikely to be due to the continued formation of an unstable *O*-acyl intermediate. The different patterns of inhibition at lower and higher concentrations of ETC could reflect the apparent consumption of the reagent at low ETC concentrations being overcome at high concentrations.

Incubation of cells with high concentrations of ETC, even in phosphate-buffered saline, is capable of producing a stable inhibition of transport. The reversal of transport inhibition is clearly dependent on the concentration of ETC that is used for treatment, and the effects of ETC concentration could contribute to the lack of consistency of the reversal of inhibition that we have observed for treatments with 18 mM ETC, and the lack of agreement between our results and others'. The finding that above 50 mM ETC a slightly higher inhibition results after 60 minutes incubation than after 5 minutes is consistent with the finding by Wieth (Wieth *et al.*, 1982a) of a fast and a slow component of inactivation by ETC.

Inhibition was also produced in a non-phosphate, non-citrate buffer, PIPES-buffered sucrose, as was mentioned briefly in Chapter IV. It would be anticipated that this inhibition occurs by the same mechanism as that in phosphate-buffered saline -- it certainly does not occur by the citrate-dependent mechanism described in Chapter IV. The amount of inhibition produced by a given ETC concentration in PIPES-buffered sucrose (listed in Table V.1) is comparable to that produced in PBS (see Figure V.2). However, the inhibition in PIPES-sucrose does not appear to be reduced by continued incubation with ETC for 60 minutes, instead, it is higher. Recall that, in PBS, the inhibition by lower concentrations of ETC (such as the 8 and 18 mM concentrations used in PIPES-buffered

TABLE V.1
INHIBITION OF ANION EXCHANGE BY ETC TREATMENT IN PIPES-BUFFERED SUCROSE

treatment	$pH_{treatment}$	$t_{1/2}$	inhibition of transport (%)
ETC, 9 mM, 5 min	6.85	(11.6)/12.6	8
ETC, 9 mM, 5 min	6.35	(12.3)/14.4	14
ETC, 9 mM, 5 min	6.30	(14.6)/17.2	15
ETC, 16 mM, 5 min	6.30	(8)/11.3	29
control, 5 min	6.38	12.5	(0)
ETC, 8 mM, 5 min	6.43	14.0	13
ETC, 18 mM, 5 min	6.59	17.1	27
control, 60 min	6.49	12.9	(0)
ETC, 8 mM, 60 min	6.58	18.5	30
ETC, 18 mM, 60 min	6.70	23.8	46

Treatment with ETC and transport measurements are described in Methods. The composition of the PIPES-buffered sucrose is 30 mM PIPES, pH 6.5, 230 mM sucrose. The pHs listed are the measured pH of the cell suspension during treatment. The top set consists of separate measurements, for which the control $t_{1/2}$ for each is listed in parentheses. The lower two sets represent one experiment. The pH of transport for all samples was within the range of 6.83 to 6.90.

sucrose) reversed over a 60 minute incubation. Thus, it appears that the buffer components, either directly or indirectly, affect the reversibility of the inhibition produced by ETC.

The pH of reaction of cells with ETC is also suggested to affect the reversibility of the inhibition produced. A full scale pH-dependence study was not done. However, a few points should make it clear that the pH dependence is distinctly different from that of treatment in citrate-buffered sucrose, which was found to increase with increasing pH over the range pH = 5 - 9.5. A hint of the pH dependence of production of inhibition was seen in the data for inhibition in PIPES-buffered sucrose. In that case, an increase of 0.5 pH units (6.35 to 6.85) resulted in a reduction of the inhibition by about one half. In MES-buffered KCl, in a suspension of cells that had been pH-adjusted such that the treatment pH was 5.73, treatment with 18 mM ETC for 60 minutes produced 47% inhibition (compare this to 10 to 22% inhibition at pH ≅ 6.5 by this concentration of ETC, estimated from Figure V.2). The enhancement of inhibition by treatment at lower pHs is to be expected for a typical carbodiimide-carboxyl reaction mechanism.

2. [^{14}C]ETC Labeling of Membrane Proteins

a. Level of Incorporation Into Ghost Membranes. To determine whether ETC was incorporated into Band 3, and perhaps label residues that might be involved in the inhibition, reaction with [^{14}C]ETC was performed. First, the rationale for the selection of conditions for labeling should be explained. The intention was to treat intact red cells, since it was known that this limits the reaction with other proteins (the permeable EDC leads to crosslinking). Conditions that would be expected to not show any incorporation were included, as well as ones in which substantial incorporation might be expected. Two cases in which we expected no incorporation were for low concentrations of ETC after 60 minutes' treatment, with or without nucleophile, in which case the inhibition reverses, and is stabilized by the nucleophile, respectively. Since treatment with high concentrations of ETC, at low pH, or in PIPES-buffered sucrose leads to substantial levels of irreversible inhibition, these conditions might be expected to result in substantial incorporation of ETC. A question that is important for understanding what leads to the irreversible inhibition in these cases is: is lack of reversal due to ETC incorporation?

Table V.2 lists the values of [^{14}C]ETC incorporation per mg of ghost membrane protein, for the different conditions under which labeling was performed. There was substantial incorporation in all cases, considering that a value of one mol per mol Band 3

TABLE V.2
INCORPORATION OF [^{14}C]ETC INTO ERYTHROCYTE MEMBRANES

trial	actual concentration ETC (mM)	pH	other treatment	[^{14}C]ETC incorporation (nmol / mg ghost protein)
1	5.5	6.5	- -	16.8
2	6.0	6.5	+ 50 mM PheOEt	10.0
3	5.0	6.3	in PIPES-sucrose	17.9
4	11.5	6.5	- -	20.4
5	11.0	5.7	(pH-adjusted cells)	17.9
6	10.7	6.5	+ 100 µM DNDS	23.6
7	35.	6.5	- -	31.2

Erythrocytes were treated with [^{14}C]ETC as described in Methods. The ETC concentrations were determined from the radioactivity in the supernatant after pelleting the cells at the end of the treatment. The pHs were determined using larger "mock" trials using the same buffers, additions and cells, and non-radioactive ETC. Ghost membranes were prepared from the treated cells, and the measured incorporation of radioactivity expressed per mg of membrane protein.

would be equivalent to about 2.5 nmol/mg membrane protein. This is distinctly more than would be expected to be incorporated into Band 3. There is also variation in the labeling among the treatments, however, it does not seem to be correlated with the level of inhibition produced by the different treatments.

The unexpectedly high level of labeling was not due to reaction with sites on the inside of the membrane. The amount of [^{14}C]ETC that was trapped in the cells was quite low. The average fractional radioactivity trapped in the cells (and released upon hemolysis) was 0.18% of the total radioactivity added for 60 minutes' incubation.

b. Incorporation Is Mostly Into Glycophorin A, Not Into Band 3. The [^{14}C]ETC-labeled proteins in ghost membranes from 3 of the 7 trials (1,4 and 7) were separated using SDS polyacrylamide gel electrophoresis. For all three, the radiolabel was found primarily in two major peaks, as indicated by the densitometric scan of the fluorogram of the gel shown in Figure V.3 (tracing B shows the sample from trial 1). The position of these peaks in radioactivity does not appear to be coincident with any of the major Coomassie Blue stained bands (the scan of the Coomassie Blue-stained gel is shown in the top panel). However, the R_fs of the bands in the fluorogram (0.33 and 0.65) compare quite closely to the R_fs of the two major bands that are stained blue with Stains-All (R_f = 0.31 and 0.67) (a scan of the Stains-All staining of sample 4 of the same gel is shown in panel D). Based on their mobility in the gel, and their staining characteristics, these two bands most likely correspond to the periodic acid-Schiff reagent-staining bands called PAS-1 and PAS-2 (with lower, and higher R_f, respectively), which are composed of a glycophorin A dimer, and monomer, respectively (Dzandu, Deh and Wise, 1985 ; King and Morrison, 1976). Thus, about 75% of the [^{14}C]ETC label in the membranes is in glycophorin A. The treatments producing higher levels of incorporation into the membrane correspondingly increased the intensity in the two glycophorin bands. The fractional intensity in the trace of the fluorogram in the region corresponding to Band 3 was not more than 8% of the total intensity. When the gel was dried without fixing or staining (trace C in Figure V.3) there was more radioactivity (11-12%) in the region of Band 3. However, this estimate would be high, since there is a non-baseline resolution of peaks in this area of the fluorogram. The estimated upper limit for the amount of ETC in Band 3 would then be about 0.9 nmol / mg membrane protein (~0.38 mol/mol), although the true stoichiometry in Band 3 may be considerably lower than this value.

c. Effect of Hydroxylamine or Trypsin Treatment of Ghosts on the [^{14}C]ETC Label. The carbodiimide adduct with tyrosine is sensitive to hydroxylamine (Carraway and Koshland, 1972). Therefore, the effect of hydroxylamine treatment on the

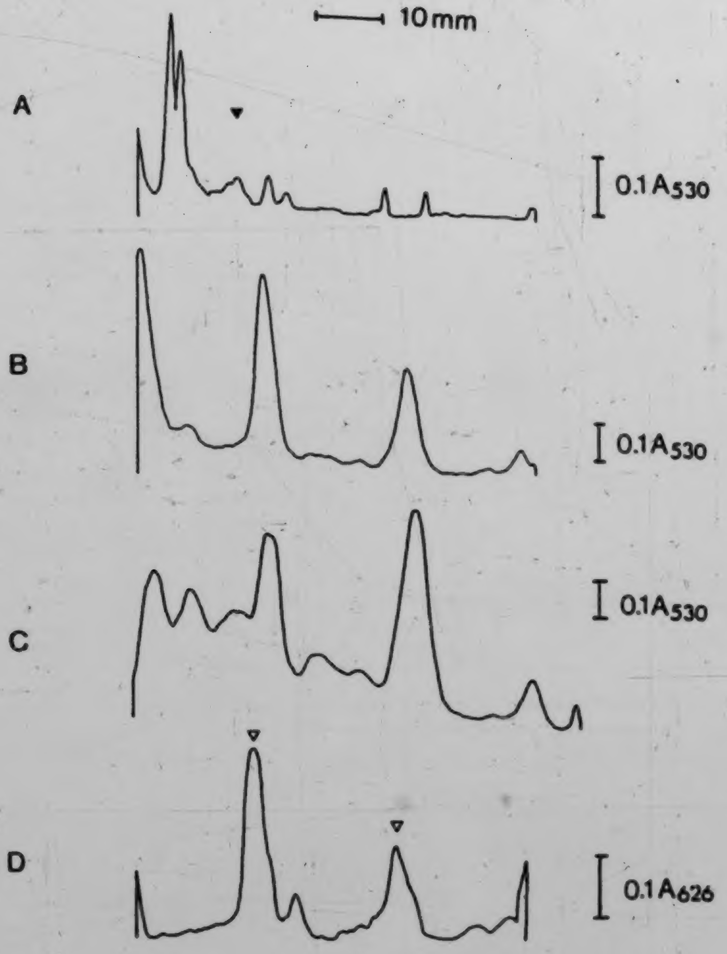

Figure V.3 Densitometric scans of the stained SDS polyacrylamide gel, and its fluorogram, showing incorporation of [^{14}C]ETC into membrane proteins. Samples of the [^{14}C]ETC-labeled ghosts were electrophoresed on an SDS polyacrylamide gel containing 8% acrylamide.The gel was stained with Coomassie Blue, except one lane, which was stained with Stains-All, then prepared for fluorography. The vertical bar indicates the absorption scale, and the horizontal bar indicates the distance from the start of the scan, in mm. A, The Coomassie Blue-stained gel of the sample from trial 1 (as listed in Table V.2) was scanned with a 530 nm filter. The arrow (▼) denotes the position of Band 3. B, The fluorogram of the gel containing sample 1 was scanned at 530 nm. C,The sample from trial 1 was electrophoresed on a separate gel, this time without fixing or staining prior to drying for autoradiography. The autoradiogram was scanned at 530 nm. D, The lane stained with Stains-All was scanned using a 626 nm filter. The peaks marked by open arrows correspond to the glycophorin A dimer (with lower mobility) and monomer (with higher mobility).

[^{14}C]ETC label would indicate whether modification of tyrosine residues had occurred. Treatment of the ghosts from trial #3 (in Table V.2) with 1 M hydroxylamine, pH 7.25, for 1 hr at room temperature released some radioactivity from the membranes (about 19% of the estimated total). This indicates that a significant amount of the label may be associated with tyrosine residues. Trypsin treatment of the ghosts (from trial #5) at a 1:4 suspension in 10 mM sodium phosphate, 150 mM KCl, pH 7.4, with 50 µg trypsin / mL suspension, for 1 hr at 37 °C, conditions expected to remove a portion of glycophorin A, released about half the radioactivity from the membranes, and that remaining associated with the membrane migrated with the dye front of a polyacrylamide gel (not shown). No other regions of labeling (including any corresponding to Band 3 trypsin fragments) were observed.

3. Stabilization of the Inhibition by Exogenous Nucleophiles

One approach to obtaining labeling and identification of the carboxyl residue(s) that are modified and are thus implicated as important for transport function is by addition of an exogenous nucleophile (in high enough concentration to compete with water, Figure IV.1,b). Carbodiimide-coupled incorporation of nucleophile has been used in many instances in other systems (for example, Hoare and Koshland, 1967; Carraway and Koshland, 1972). Reaction of intact cells with ETC was done in the presence of several amines (taurine, ethanolamine and hydroxylamine), and a series of amino acid ethyl esters in which the α-carboxyl is esterified to prevent activation by ETC, and the α-NH_2 is free to act as a nucleophile. Treatment of cells with the nucleophiles alone did not cause inhibition. At short times of treatment (5 min), for all nucleophiles tested, inhibition after treatment with ETC plus nucleophile was not substantially enhanced compared to reaction without exogenous nucleophile. However, for some nucleophiles, inhibition remained after treatment for longer times (up to 120 min). The time course of inhibition by ETC in PBS, with and without the added nucleophile PheOEt, is shown in Figure VI.1. Without added nucleophile the inhibition reversed almost fully by 60 min. With added PheOEt (50 mM) inhibition was clearly stabilized, remaining at the level (35% inhibiton) that is reached within 2 minutes of reaction for up to 120 minutes.

Table V.3 lists the level of inhibition, relative to control, remaining after a 60 minute incubation with ETC plus several nucleophiles[3]. The ability to afford stabilization of the inhibition to reversal appeared to be related to the hydrophobicity of the nucleophile, at least for the series of amino acid ethyl esters. Note that glycine ethyl ester did not provide stabilization, in agreement with what Craik and Reithmeier found previously (1985). The observation of stabilization of inhibition by addition of a nucleophile further supports that the reaction of ETC in saline media is with a carboxyl residue, since it is only this adduct that can be displaced by addition of a nucleophile (Hoare and Koshland, 1967; Carraway and Koshland, 1972). Further, it appears that modification of the carboxyl group(s), either by the carbodiimide isourea intermediate, or by the formation of an amide with an exogenous nucleophile, alone is enough to cause inhibiton of anion exchange.

Two other potential nucleophiles, which are not listed in Table V.3, were tested, N-hydroxysuccinimide and N-hydroxysulfosuccinimide. It was thought that the use of these compounds, which might be expected to form a relatively stable active ester with the protein, would allow isolation of the protein and subsequent reaction with a nucleophile. Alone, these reagents both produced some inhibition of transport, from 0 to 12%, when the washing procedure used for the other nucleophiles was followed. However, treatment with these reagents in combination with 18 mM ETC resulted in either an absence of inhibition by the ETC, or a transport rate that was from 10% to 35% faster than that in the

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[3] In using this approach to screen nucleophiles for their suitability in a labeling experiment we are assuming that if a nucleophile forms an adduct with the carboxyl, then the transport would be inhibited, in a manner similar to the inhibition by the carbodiimide adduct. This is not always a safe assumption, since some modifications have left catalytic activity intact (Means and Feeney, 1971).

Table V.3

Inhibition of anion exchange after 60 minutes'
treatment with ETC in PBS in the presence of added nucleophile

nucleophile	% inhibition at 60 min	(# det'ns)
taurine	0	(2)
ethanolamine	3	(3)
hydroxylamine	28	(1)
GlyOEt	3	(2)
AlaOEt	0	(2)
MetOEt	8	(4)
NTyrOEt	12	(1)
TyrOEt	17	(2)
LeuOEt	16	(1)
PheOEt	35	(6; s.d.=7.3)

The nucleophiles used included a series of amino acid ethyl esters (abbreviated AAOEt). A nucleophile concentration of 50 mM was used, except for ethanolamine, which was present at 10 mM, and hydroxylamine, which was present at 30 mM. These concentrations of nucleophiles did not cause inhibition without added ETC, provided that washes, as outlined in Methods, preceded the transport measurement. Treatment was as described in Figure V.1, but for 60 minutes. The % inhibition is relative to that for ETC alone after 60 minutes.

untreated control cells. These reagents did not seem to affect the production of the usual level of inhibition by ETC plus PheOEt. We do not have a good explanation for this interesting result.

The dependence on the ETC concentration of the ability of the nucleophile PheOEt to provide inhibition was determined, since it was shown that lower concentrations of ETC provide a more distinct difference between inhibition by ETC alone at 5 minutes compared to 60 minutes (a more distinct reversal). Even at 5 mM ETC, PheOEt still provides inhibition that remains after 60 minutes' incubation (34%, with perhaps 3 to 14% due to ETC alone). 1 mM ETC is not sufficient, since only 3% inhibition was produced by incubation of 1 mM ETC plus 50 mM PheOEt with cells for 60 minutes.

4. ETC-Mediated Incorporation of [^{14}C]PheOEt Into Membrane Proteins

a. Selection of Conditions for Labeling. Conditions that optimize carbodiimide mediated incorporation of nucleophile into a protein have most often been defined for soluble proteins (Hoare and Koshland, 1967; Carraway and Koshland, 1972), and usually involve using high concentrations of the nucleophile (0.1 M). The results obtained with nucleophile stabilization of inhibition of anion exchange indicate that the higher nucleophile concentrations tested (50 mM) increase the efficiency of, and may be absolutely necessary for, the stabilization effect (data not shown). The use of even higher concentrations of nucleophiles is limited by the need to maintain the permeability barrier of the cells. Use of concentrations of 65 mM and higher resulted in substantial hemolysis (data not shown). An attempt at treating with higher nucleophile concentrations was made using erythrocyte ghosts. Treatment of ghost membranes with 1 mM ETC (without nucleophile) led to substantial crosslinking of the membrane proteins, such that a band in the Band 3 region of an SDS polycrylamide gel could no longer be distinguished. Clearly, this would not be of much use in trying to identify labeled proteins. Thus, the conditions chosen to achieve measurable labeling were those that consistently produced about 35 % inhibition, using the nucleophile PheOEt at 50 mM with 18 mM ETC.

b. Labeling of Membrane Proteins by Treatment of Red Cells With ETC and [^{14}C]PheOEt. [^{14}C]PheOEt was synthesized from [^{14}C]Phe as described in Methods. A preliminary attempt (not shown) at incorporation of [^{14}C]PheOEt showed incorporation into the following proteins : a small, but distinct amount of label in the Band 3 region; approximately twice as much in a band just below Band 3 (presumably PAS-1);

two bands below this, each with about the same amount of radioactivity as in Band 3 (the lower one might correspond to PAS-2); and a band with an R_f of 0.87, with approximately 6 times the level of incorporation as Band 3. The incorporation into Band 3 was estimated to represent about 0.2 mol per mol Band 3, but accurate quantitation was not done. This value is somewhat lower than the 35% inhibition produced under similar conditions. The low stoichiometry would be a problem, and would limit further analysis of the labeling.

In an attempt to repeat this experiment, three aliquots of cells were successively treated with [^{14}C]PheOEt , one with no proteolysis, one in which the cells were treated with α-chymotrypsin, and one in which the ghosts were treated with trypsin. All three had similar levels of total incorporation of radioactivity into the membranes: 3.7, 4.7 and 4.7 nmol [^{14}C]PheOEt incorporated per mg membrane protein. However, the major bands of labeling (in the un-proteolyzed cells) co-migrated with PAS-1 and PAS-2, and likely correspond to glycophorin A_2 and glycophorin A (see the section on [^{14}C]ETC labeling for more detail on this assignment). The labeling after chymotrypsin fragmentation is also consistent with incorporation into the glycophorins. The original bands at PAS-1 and PAS-2 positions are reduced in intensity, and a labeled band corresponding to the chymotrypsin fragment of glycophorin A_2 (Dzandu, Deh and Wise, 1985) appears -- it stains blue with Stains-All, and migrates slightly above the red-staining band corresponding to the 60 kDa Band 3 fragment . In addition, an unidentified band of higher mobility also appears.

Trypsin treatment of ghosts resulted in reduction in the amount of the two bands seen in the un-proteolyzed sample, and the appearance of two new bands that did not correspond with the mobilities expected for the known fragments of glycophorin produced by trypsin treatment of cells (Dzandu, Deh and Wise, 1985), yet were not extensively reduced in size (as expected for proteolysis of unsealed ghosts). Clearly, the [^{14}C]PheOEt label is not as completely removed by trypsinization of ghosts as is the [^{14}C]ETC treatment

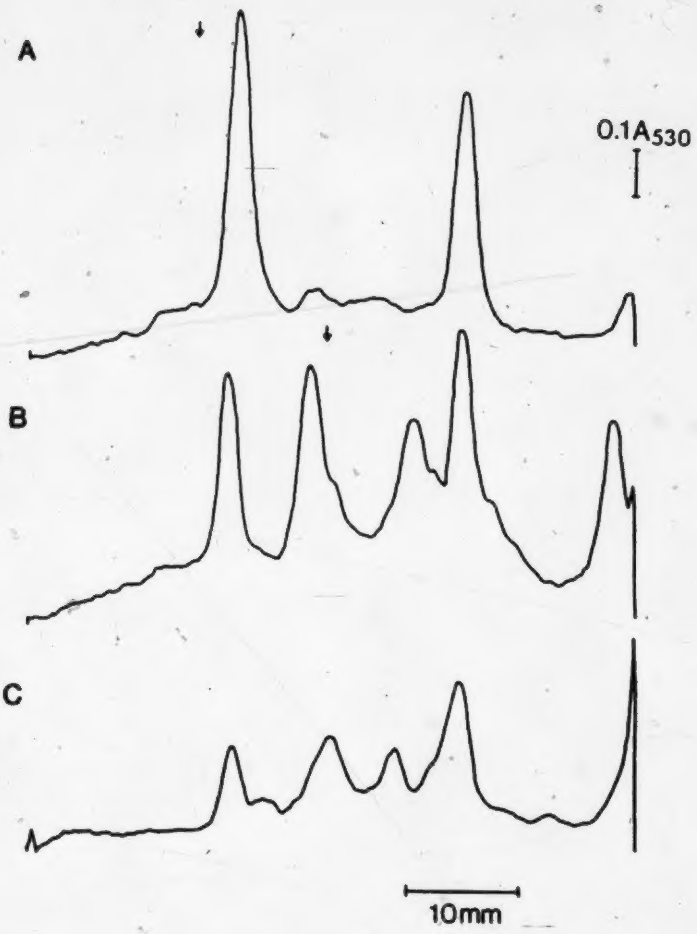

Figure V.4 Densitometric scans of the fluorogram of [^{14}C]PheOEt labeled membrane proteins. The labeled ghosts were electrophoresed on an 8% SDS polyacrylamide gel, which was fixed and submitted to fluorography as described in Methods. The densitometric scans for three samples are shown: A, no proteolysis, in which the arrow indicates the migration of Coomassie Blue-stained Band 3; B, chymotrypsin-treated cells, in which the arrow indicates the migration of Coomassie Blue-stained 60 kDa chymotryptic fragment of Band 3; C, trypsin-treated ghosts.

that was described earlier (section V.C.2). It is possible that both represent incorporation into the same protein, but into a slightly different set of residues. Although essentially the same conditions were used in the treatment described above, the incorporation into Band 3 was not reproducible.

5. Relation to the Stilbenedisulfonate Binding Site

a. Effect of Inhibitory Treatment with ETC on $^3H_2$DIDS Labeling of Band 3. To test whether the $^3H_2$DIDS covalent reaction sites are intact after reaction of red cells with ETC in saline media, $^3H_2$DIDS labeling measurements were carried out on untreated cells, and on cells that had been treated with 18 mM ETC in PBS. Values of 2.43 nmol $^3H_2$DIDS / mg membrane protein for ETC-treated, and 2.22 nmol / mg membrane protein for untreated compare well with the previously obtained values for labeling in untreated cells (see Chapter IV), indicating that the covalent reaction with $^3H_2$DIDS remains unaffected by inhibitory treatment with ETC in saline media. Under these conditions of ETC treatment we observe 20 to 25% inhibition of anion transport . We would expect to see about the same reduction in labeling of Band 3 by $^3H_2$DIDS if, for instance, lysine *a* or other stilbenedisulfonate binding site residues were involved.

b. Effect of DNDS on the Production of Transport Inhibition by ETC. When 100 μM DNDS was included in the reaction mixture of cells with ETC the inhibition produced appeared to be somewhat enhanced, as shown by the data in Table V.4. At both a relatively low concentration of ETC (7 mM), and a relatively high concentration (50 mM), treated for either 5 minutes or 60 minutes, the inhibition is higher in the presence of DNDS. All the values of inhibition in the absence of DNDS are lower than expected, compared with those in Figure V.2, but this might be partly explained by the fact that in order to reduce the residual inhibition by DNDS alone, it was necessary to perform washes at room

TABLE V.4

EFFECT OF DNDS ON THE PRODUCTION OF TRANSPORT INHIBITION BY ETC

conditions	transport inhibition (%)
DNDS alone (100 µM)	2
ETC (7 mM), 5 min	0
ETC (7 mM), 5 min [a]	6
ETC (7 mm) + DNDS (100 µM), 5 min	13
DNDS alone (100 µM)	0
ETC (50 mM), 5 min	6
ETC (50 mM) + DNDS (100 µM), 5 min	17
ETC (50 mM), 60 min	25
ETC (50 mM) + DNDS (100 µM), 60 min	51

[a] washes were all at 0 to 4 °C, unlike the washing procedure described in Methods for all others, in which the first 3 washes were at room temperature.

temperature, during which time the inhibition may be reversing. Even accepting this discrepancy, when the data was considered within the context of this experiment, DNDS clearly does not prevent inhibition by ETC in these conditions.

6. Inhibition of Anion Transport in Mouse Red Cells

As an approach to limiting the carboxyl residues which might be involved in the inhibition, the species differences between the murine and human Band 3 protein sequences were used. The inhibition of transport in mouse red cells by ETC was studied. A concentration of 20 mM ETC produced 24% inhibition in MES-buffered saline (5 minutes treatment, pH, treatment = 7.0). When treated for 5 minutes in MES-buffered saline, washed, then incubated 60 minutes more in MES-saline alone, inhibition was 28% in one sample, and 17% in another. The levels of inhibition produced by this concentration of ETC are quite comparable to those produced in human cells. One possible difference, though, is in the apparent lack of reversal of the inhibition produced in MES-saline over a 60 minute incubation. Inspection of the murine deduced protein sequence, and comparison with the segments in which the sequence of the human protein is known, reveals a difference in the location of carboxylate side-chain residues. In the segment equivalent to ChPa 8, the murine sequence has Asp-625 and Asp-639, but not Asp-644 in the equivalent positions as the human sequence. One can conclude that the carboxylate-mode of inhibition by ETC must not be acting _via_ Asp-644, since anion exchange in mouse red cells, in which Band 3 lacks an Asp near 644, was inhibited by ETC in MES-saline.

D. DISCUSSION

Unlike the inhibition produced by ETC in the presence of citrate, the inhibition produced in the absence of citrate appears to be due to the modification of protein carboxyl residues. Our results of reversible inhibition that is stabilized by the addition of certain nucleophiles are strong evidence for the involvement of a carboxyl residue(s) in anion exchange. Based on known carbodiimide chemistry, it is only a carboxyl adduct which can be affected by addition of a nucleophile (Hoare and Koshland, 1967). The results also suggest that both modifications, either the carbodiimide-carboxyl intermediate or the nucleophile adduct, can produce inhibition. This is interesting, since the ETC-adduct would be positively charged, while the nucleophile adducts would be uncharged (except taurine), although bulky. The finding that the phenomenon of reversal of the inhibition is not consistently produced under all conditions does not seriously dampen this conclusion. However, in the absence of demonstration of incorporation of significant amounts of nucleophile into Band 3, it should be considered that the effect of nucleophiles on the inhibition could be due to a less straightforward mechanism, perhaps similar to the effect of other buffer constituents on the reversibility of the inhibition.

The lack of reproducible reversal of the inhibition is disturbing and forces us to consider other possibilities to explain the phenomenon of reversal. Another possible explanation of reversal is that the reagent inhibits by noncovalent binding, and that the binding is so tight that release only occurs over a time course of an hour. If one were to measure release of [^{14}C]ETC from the membranes, this possibility would be indistinguishable experimentally from one involving hydrolysis of the *O*-acylisourea adduct. However, considering that nucleophiles stabilize the inhibition, the hydrolysis is the more plausible explanation. Only certain conditions allowed manifestation of the reversible type of inhibition. The reversible component was not observed in PIPES-buffered sucrose, or in MES-buffered NaCl (for mouse cells). Even in PBS, where there seems to be both reversible and irreversible types of inhibition, the reversible component was favored by treatment with ETC at higher pH (neutral, rather than slightly acidic). In model reactions, more alkaline pH promotes hydrolysis of the *O*-acylisourea intermediate (Hoare and Koshland, 1967). It seems a plausible possibility that treatment in phosphate-buffered saline could promote the reactions favoring hydrolysis of the carbodiimide adduct to the (unreactive) urea, either by promoting hydrolysis of the intermediate, or by effectively reducing the concentration of ETC available to react with Band 3 by side-reaction with phosphate. Either situation could create the phenomenon of reversal by producing a decreased concentration of the unstable intermediate for conversion to some stable adduct.

The nature of the irreversible inhibition produced under some conditions (in the absence of citrate) is not clearly known. The possibility that it is due to continual reaction with excess free reagent was shown to be unlikely by the lack of reversibility of inhibition produced by treatment with 50 mM ETC for 5 minutes in PBS. The other possibilities are, of course, those considered in the general reaction scheme of carbodiimides, shown in

Figure IV.1. Stable incorporation of the carbodiimide into an amino acid residue is a possibility, as is nucleophilic displacement by an endogenous nucleophile. The possible rate of occurrence of these last two possibilities would be sensitive to the concentration of the *O*-acylisourea intermediate, which would be sensitive to the effective concentration of ETC.

We cannot unequivocally rule out a lack of incorporation of [^{14}C]ETC into Band 3 under citrate-free conditions, as had been demonstrated by Craik (1985) in citrate-buffered sucrose. The several reaction conditions tested produced detectable, but not quantifiable (by the method of SDS gel electrophoresis and fluorography), incorporation into the region of Band 3. However, the conditions that produced irreversible inhibition did not systematically result in more incorporation of ETC than did those producing little (or reversible) inhibition. Additional work to determine the extent of incorporation of [^{14}C]ETC into Band 3 should be done, perhaps by isolation of the labeled protein by immunoaffinity methods. The labeling of glycophorin by ETC (and by PheOEt, mediated by ETC) can be presumed to be a side-effect, unrelated to the inhibition of anion exchange by ETC, since it is already firmly established that Band 3 is solely responsible for the distinctive anion exchange (and that glycophorin is not involved). The site of attachment of ETC in glycophorin may be of interest since it defines a reactive extracellular carboxyl group.

The identification of the particular residues that are modified in Band 3 by treatment with ETC in saline is still unknown. We can speculate that the region of the protein surrounding these residues is hydrophobic since the more hydrophobic nucleophiles tested provided stabilization, while the more hydrophilic ones (excepting hydroxylamine) did not (we are assuming that if an adduct is formed, it will be inhibitory). Whatever the identity of the residues that are involved in the production of inhibition, they are not in the stilbenedisulfonate binding site, since reaction in the presence of DNDS did not reduce the inhibition.

Our attempts at labeling Band 3 with $[^{14}C]$PheOEt to localize and identify the modified carboxyl(s) resulted in a very low stoichiometry of labeling, and may limit the use of this approach to identifying the residues involved in inhibition of transport by ETC. Our finding that stilbenedisulfonates do not protect against the inhibition limits our use of these inhibitors to protect the Band 3 residues from reaction in a pre-reaction of the apparently very reactive glycophorin residues. It is especially concerning that we could not obtain labeling with PheOEt since there had been an earlier, brief report from another lab (Bjerrum, 1983) that suggested they had achieved stoichiometric incorporation of TyrOEt into Band 3, possibly located in the ChPa-8 fragment.

There are additional discrepancies between the data that we have obtained, and the data reported by Wieth and co-workers, using the same reagent. These discrepancies make it unclear whether the mode of inhibition that we report for treatment in citrate-free media is the same as that reported by their lab. Since in Wieth's work the treatment with 30 mM ETC was performed in 165 mM KCl, 5 mM 2-(N-morpholino)-ethanesulfonic acid, at pH 6, the results should be comparable to those for our treatments with 18 mM ETC in PBS, pH 6.8. In fact, we can confirm their results of the production of about 35% inhibition with ETC plus a hydrophobic nucleophile (though not with the same one). Differences between their findings and ours are: 1, they do not report any evidence of decay of the initially irreversible inhibition in the absence of added nucleophile, as we have observed under some conditions, although we find less reversal at high (30 mM) ETC concentrations; 2, they observed an accelerated reaction rate in the presence of tyrosine ethyl ester, which we did not distinguish; and 3, they observed an apparent loss of covalent DIDS binding[4], while we observed no reduction in H_2DIDS labeling after treatment with ETC in the absence of citrate.

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[4] Their observation was actually that only half the 'stoichiometric' number of covalently bound DIDS molecules were required to further inhibit transport to 100% in cells that have been inhibited by ETC to 50%. This implies that the modified Band 3 molecules are not reacting with DIDS.

We can only attempt to understand these differences by noting that there are small differences in the treatment conditions: they "stopped" their reaction by the addition of a cold solution containing tris-(hydroxymethyl)amino methane, pH 9 (a potential nucleophile); and the pH difference of 0.8 pH units may be significant, since we found that at pH 5.7 there was not a reversal of inhibition with a 60 minutes incubation. Both of these differences may make the results less comparable, especially regarding reversal. Additionally, they measured transport activity by Cl^--Cl^- exchange, as efflux from resealed ghosts, and we measured P_i-limited influx into Cl^--loaded cells. This difference is perhaps the most significant, since it is possible that a modification may have different effects on P_i and Cl^- transport, or on the inward and outward steps of the transport. Alteration of Band 3 protein by limited proteolysis with papain is known to have different effects on the outward and inward rate constants of the transport (Jennings and Adams, 1981). The relationship between their tentative location, using tyrosine ethyl ester, of the labeled residue to the 35 kDa fragment (Bjerrum, 1983) and our work can be considered only after the discrepancies in the characteristics of the inhibition are clarified.

The results using another chemical modifying reagent have been much more clear than those using carbodiimides. Using a different method for labeling carboxyl groups, Woodward's Reagent K followed by $NaBH_4$, Jennings' group recently reported that the modification of at least two glutamate residues near the stilbenedisulfonate site results in greater than 90% inhibition of chloride-bromide exchange (Jennings and Anderson, 1987). More recent results characterize the effects of treatment that produces the corresponding carbinols of the carboxylates (Jennings, 1988; Jennings, personal communication). Band 3 modified in this way has stimulated sulfate influx, but no longer cotransports a proton with sulfate in SO_4-Cl exchange. In other words, its transport behavior is that expected for the protein in which the residue thought to provide the titratable site that binds and cotransports a proton with sulfate is permanently protonated.

As Jennings and Anderson (1987) point out, Woodward's Reagent K and ETC might be expected to react with different sets of residues in Band 3 since the two reagents are oppositely charged (Woodward's Reagent K is negatively charged, while ETC is positively charged). Comparison of the characteristics of the inhibition produced by Woodward's Reagent K treatment to that by the ETC treatment clearly indicates that the residues involved are not the same. The inhibition by ETC is not prevented by DNDS (our results, and Borders, C.L., personal communication), while the Woodward's Reagent K modification and inhibition were prevented by the presence of DNDS. One of the glutamate residues modified by Woodward's Reagent K corresponds to the residue involved in proton cotransport with sulfate (Jennings, submitted), and may be involved in forming part of the permebility barrier (Jennings, 1988). In support of a potential functional importance of carboxylates outside the stilbenedisulfonate binding site, it should be noted that the low pK "regulator sites" responsible for the pH dependence of anion transport have been found by some to lie outside the stilbene-spin probe binding site (Kaufmann, Eberl and Schnell, 1986).

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VI. REACTIVITY AND ACCESSIBILITY OF THE N-ETHYLMALEIMIDE-UNREACTIVE SULFHYDRYL IN BAND 3 PROTEIN[1]

A. INTRODUCTION

Estimates of the cysteine content of purified human erythrocyte Band 3 protein range from five to nine moles per mole protein (Ho and Guidotti, 1975; Jenkins and Tanner, 1977; Drickamer, 1977; Fukada *et al.*, 1978; Steck *et al.*, 1978), with the commonly accepted value being 6 cysteine residues. This content is based on amino acid analyses after performic acid oxidation of the purified protein. Two reports suggest greater than 6 residues: 9.7 mol/1000 mol amino acid (Ho and Guidotti, 1975), and 8.5 ± 1.5 mol/1000 mol amino acid (Jenkins and Tanner, 1977). Three reports indicate values of 5 to 6 mol cysteine/mol Band 3 (e.g., the value in Steck *et al.* is 5.8 ± 0.8 mol/mol) (Drickamer, 1977; Fukada *et al.*, 1978; Steck *et al.*, 1978).

The protein sequence deduced from a mouse Band 3 cDNA (Kopito and Lodish, 1985) contains six cysteine residues, however, at least one of these, Cys-152, is not found in the corresponding human protein sequence (Kaul *et al.*, 1983). This difference is supported by the sequence deduced from a cDNA clone of human Band 3, which contains five cysteine residues, located in approximately the same positions as those in the murine sequence, except lacking the one corresponding to murine Cys-152 (Lux and Lodish, personal communication) (a summary of the relative locations of the cysteine residues is shown in Figure VI.1). The absence of the cysteine residue corresponding to murine Cys-152 in the sequence of the human protein is also in part supported by the values for Cys in the amino acid analyses of fragments of human Band 3 containing this part of the sequence (Drickamer, 1977; Steck *et al.*, 1978), and by the absence of NEM labeling of the corresponding proteolytic fragment (NTr-23) (Rao and Reithmeier, 1979).

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[1] A version of this chapter has been submitted for publication: Werner, P.K. and Reithmeier, R.A.F. (1988) Biochim. Biophys. Acta .

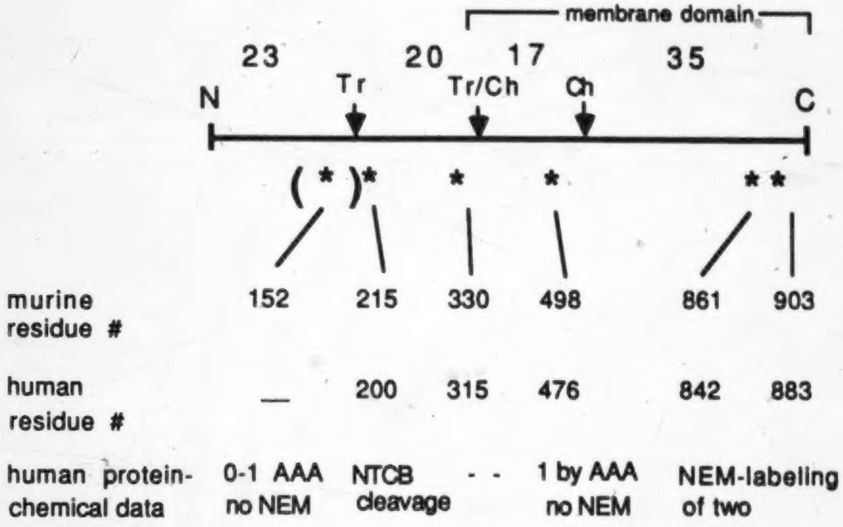
membrane domain
23
20
17
35
N
Tr
Tr/Ch
Ch
C
(*)*
*
*
* *
murine residue #
152
215
330
498
861
903
human residue #
—
200
315
476
842
883
human protein-chemical data
0-1 AAA no NEM
NTCB cleavage
- -
1 by AAA no NEM
NEM-labeling of two

Figure VI.1 Summary of the relative locations of the cysteine residues in the sequences of murine and human Band 3 protein. The locations of cysteine residues within the sequence, relative to the major proteolytic cleavage sites (indicated by arrows), are indicated by asterisks (*), with the exact residue numbers indicated for the murine sequence (Kopito and Lodish, 1985), and the human sequence (Lux and Lodish, personal communication). The protein-chemical data from the human protein relating to the presence and location of sulfhydryls is also summarized. AAA refers to determinations by amino acid analysis for cysteine content. The notation of 'no NEM' indicates there is no NEM incorporation into fragments containing this region. Abbreviations for the cleavages are defined in Figure I.2.

Generation of the 23 kDa N-terminal fragment (Kaul *et al.*, 1983) by NTCB cleavage is consistent with the same relative location of murine Cys-215 in the human sequence. The available protein sequence data from the human protein does not overlap the other 4 cysteine residues, however, the results of amino acid analysis (for 498) or NEM labeling (for 842 and 883) support the presence of these residues in the same general locations.

All but one of the cysteine residues in human Band 3 can be modified in cells with either NEM or pCMBS, with a stoichiometry of NEM labeling of 5 mol/mol Band 3 (Rao, 1979). All these NEM-reactive residues are located on the cytoplasmic side of the membrane (Rao, 1979; Ramjeesingh *et al.*, 1983) three being located in the 20 kDa tryptic fragment of the N-terminal cytoplasmic domain (Rao and Reithmeier, 1979) and two within a 9 kDa section of the C-terminal 35 kDa fragment (Rao, 1979; Ramjeesingh *et al.*, 1981). The two sulfhydryls in the C-terminal fragment do not react with NEM in ghosts unless the ghosts are first reduced with high concentrations of 2-mercaptoethanol (Rao, 1979), but can be labeled with NEM in inside-out vesicles (Ramjeesingh *et al.*, 1981)). The five residues in human Band 3 that react during NEM treatment of cells were reported to be cysteine residues, based on determination of the amount of S-succinylcysteine (Rao, 1979). As discussed below, however, Rao's determinations (1979) must have overestimated the amount of S-succinylcysteine, and not detected some reaction of NEM with other amino acid residues, since one of the five cysteine residues is clearly not modified by NEM. Side reactions of NEM with lysine residues are a definite possibility, while the reagents pCMB and DTNB are generally thought to be specific for sulfhydryl residues (Means and Feeney, 1971).

Protein-chemical data supporting the existence of the cysteine residue corresponding to murine Cys-498, which would be located in the 17 kDa fragment, is provided both by the amino acid compositions of the fragments (Steck *et al.*, 1978; Rao and Reithmeier, 1979; Drickamer, 1977), and the cleavage with NTCB (Drickamer, 1977; Ramjeesingh *et al.*, 1980; Ramjeesingh *et al.*, 1983). This cysteine residue is clearly unreactive to NEM, since there was no [^{3}H]NEM found associated with the 17 kDa fragment after NEM treatment in cells or in ghosts (Rao and Reithmeier, 1979; Ramjeesingh, Gaarn and Rothstein, 1981). Because of its unreactivity with NEM, the cysteine residue in the 17 kDa fragment is thus denoted the "cryptic" sulfhydryl by Solomon (Solomon *et al.*, 1983). Thus, although the stiochiometries indicate five cysteine residues have reacted after NEM treatment of cells, other protein chemical evidence, in combination with the sequence data, indicate there is an NEM-unreactive cysteine residue in human Band 3.

Although unreactive with NEM, this cysteine residue appears to react with organic mercurials since, after quantitative reaction of erythrocytes with NEM, treatment with either [^{14}C]pCMB (Sha'afi and Feinstein, 1977) or [^{203}Hg]pCMBS (Solomon *et al.*, 1983; Benga *et al.*, 1986a; Benga *et al.*,1986b) resulted in association of radiolabel with Band 3 in SDS polyacrylamide gels. The site of attachment of these mercurials to Band 3 has not, however, been determined.

While pCMBS treatment of erythrocytes does not alter the rate of sulfate efflux (Knauf and Rothstein, 1981), there is a substantial volume of work suggesting that the binding of pCMBS and, in some cases, other mercurials or DTNB, to the NEM-unreactive sulfhydryl group in Band 3 inhibits the water permeability of the erythrocyte membrane (for example, Solomon *et al.*, 1983; Sha'afi and Feinstein, 1977; Macey and Farmer, 1970; Brown *et al.*, 1975; Lukacovic *et al.*, 1984; Yoon *et al.*, 1984). Furthermore, some of this evidence has been used to develop a model (Solomon *et al.*, 1983) in which Band 3 provides an aqueous channel in the red cell membrane that allows the permeation of water and urea, and controls the passage of cations and anions. The model also proposes that the NEM-unreactive sulfhydryl is located within ≅10-15 Å of the external face of the membrane, to the external side of the DIDS site. This aspect of the model is based on evidence that pCMBS is transported by Band 3 and that this transport is inhibited 98% by DIDS (Rothstein, 1981), yet DIDS does not prevent pCMBS inhibition of urea and water transport (Solomon *et al.*, 1983).

Despite the bulk of evidence appearing to relate inhibition of water permeability to a residue on Band 3 protein, there is still considerable uncertainty about whether the pCMBS interaction site that is involved in water transport inhibition is equivalent to the pCMBS site on Band 3 protein, and as to what residue on Band 3 provides the pCMBS site (Benga *et al.*, 1986a; Benga *et al.*,1986b; Verkman *et al.*, 1986a; Toon *et al.*, 1985; Toon and Solomon, 1986; Macey, 1984; Pitterich and Lawaczeck, 1985; Verkman *et al.*, 1986b). pCMBS labeling of Band 3 in SDS gels was correlated with the inhibitory effects in only some of the studies (Benga *et al.*, 1986a; Benga *et al.*,1986b), and these indicated labeling not only of Band 3, but also of band 4.5 proteins. Other studies, which employ the pCMBS-induced quenching of membrane protein tryptophan fluorescence (Solomon *et al.*, 1983; Verkman *et al.*, 1986a), or use altered binding kinetics of 4,4'-dibenzamidostilbene-2,2'-disulfonate (Lukacovic *et al.*, 1984), as a measure of pCMBS binding, provide only an indirect measure of pCMBS binding. The pCMBS labeling site of Band 3 and the water inhibition site share the qualities of being unreactive to NEM and reactive to pCMB(S), but the existing pharmacologic, kinetic and radiation inactivation evidence indicate that the water permeation pathway is distinct from the anion exchange pathway (Macey, 1984; Pitterich and Lawaczeck, 1985; Verkman *et al.*, 1986b; Jones and Frohlich, 1984). This, however, does not exclude the possibility that they are mediated by separate parts of the same protein, or perhaps by different oligomeric states of the protein.

In this chapter we describe an investigation of the reactivity of the NEM-unreactive cysteine residue in Band 3. The reaction with free sulfhydryl reagents and the interaction with a sulfhydryl affinity resin having pCMB as a ligand with detergent-solubilized, NEM-blocked Band 3[2] were studied.

B. METHODS

1. Production of NEM-Blocked Band 3

Treatment of intact erythrocytes with NEM was performed as described by Rao (1979), exposing washed erythrocytes at 25% suspension in 0.15 M NaCl, 10 mM Tris, pH 7.4, to 12 mM (final concentration) NEM for 1 hr at 37 °C. In some preparations cells were treated twice with NEM to ensure blocking of all NEM-reactive groups. Treatment of erythrocytes with [^{3}H]NEM was performed under the same conditions as for unlabeled

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[2] Throughout this chapter the phrase NEM-blocked Band 3 is used to refer to preparations of Band 3 originating from erythrocytes that have been treated with NEM.

NEM. [^{3}H]NEM at a specific radioactivity of 120 mCi/mmol in pentane (New England Nuclear) was evaporated in the bottom of a plastic tube, then dissolved in a solution of known NEM concentration, resulting in a specific radioactivity of from 4 to 8 mCi/mmol, based on the radioactivity in this stock. Calculations of the stoichiometry of labeling were based on the protein concentration in ghost suspensions, or of purified Band 3, as determined by the Lowry method (Lowry *et al.*, 1951). It has been shown that the protein determination by the Lowry method agrees quite well with protein concentrations determined by amino acid analyses, with a conversion factor for the Lowry results of 0.98 (Oikawa *et al.*, 1985).

2. Reaction of Purified Band 3 with Sulfhydryl Reagents

Purification of NEM-blocked Band 3 was performed by aminoethyl-Sepharose chromatography, as described in Chapter II. Reaction of purified Band 3 with [^{3}H]NEM in solution was carried out by diluting a freshly prepared stock of 100 mM [^{3}H]NEM (prepared as described above, with a specific radioactivity of 8.2 mCi/mmol) to final concentrations of from 0.05 to 1 mM into a solution of 1 mg/mL Band 3 in 50 mM sodium phosphate, pH 7.0, 0.1% $C_{12}E_8$. Reaction was allowed to proceed for 1 hr at 37 °C, or for 2 hr at rom temperature, as indicated. The unreacted NEM was removed by gel filtration on a Sephacryl S-200 or S-300 column in 50 mM sodium phosphate, pH 7.0, 0.1% $C_{12}E_8$. Separation of the protein-bound and unreacted radioactivity was achieved, and any contribution of free radioactivity was subtracted from that in the protein peak before calculation of the stoichiometry of labeling. Measurement of incorporation into proteolytic fragments of Band 3 was made in some cases, isolating the major proteolytic fragments NCh-60 and TrC-55 as described previously (Oikawa, Lieberman and Reithmeier, 1985). The estimate for TrCh-17 was made from the radioactivity associated with the fragment after SDS gel electrophoresis.

A stock of about 5 mM [^{14}C]pCMB was prepared by dissolving 50 µCi [^{14}C]pCMB (Research Products International) in 0.65 mL 50 mM NaCl, alkalinizing the solution with 30 µL 1 N NaOH, and neutralizing with 10 µL 1 N HCl to a pH of about 8. The specific radioactivity of 36,300 cpm/nmol (about 16 nCi/nmol) was determined from dilutions of this stock into 50 mM sodium phosphate, pH 7.0, or into 0.5 M sodium acetate, pH 4.0, using the known extinction coefficients of pCMB (Glazer *et al.*, 1975). Band 3 in solution at 1 mg/mL in 50 mM sodium phosphate, pH 7.0, 0.1% $C_{12}E_8$ was treated with 175 µM [^{14}C]pCMB for 2 hr at room temperature. These conditions avoid reaction at excessive concentrations of pCMB, and were characterized by Ojcius and Solomon (personal communication) to result in filling of both the water and urea transport inhibition sites, and in labeling of Band 3 of about 1.3 mol/mol, during treatment of erythrocytes. Separation of unreacted pCMB from labeled Band 3 was achieved in three ways: by dialysis, ultrafiltration, or gel filtration.

Measurement of DTNB reaction with ghost proteins was performed as described by Toon *et al.* (1985), using a freshly dissolved stock of DTNB and a concentration (0.5 mM) they found produced maximal reaction. The reaction was measured by determining the concentration of 3-carboxylato-4-nitrothiophenolate produced, using the extinction coefficient at 412 nm of 13,600 M^{-1} cm^{-1} (Glazer *et al.*, 1975). Reaction of DTNB with purified Band 3 in detergent solution was performed similarly, following the protocol in Glazer (Glazer *et al.*, 1975), using a 20-fold molar excess of DTNB over protein (400 µM for about 20 µM Band 3), and correcting for absorbance of the protein solution.

3. Sulfhydryl Affinity Chromatography

The interaction of NEM-blocked erythrocyte ghost proteins, or purified Band 3, with pCMB-Sepharose was performed both in columns, and in batch. The pCMB-Sepharose had been prepared by carbodiimide activated coupling of pCMB onto aminoethyl Sepharose, as described in Lukacovic *et al.* (1981). It should be noted that both sets of conditions are designed to apply a sub-saturating level of protein to the resin, which has a binding capacity of 7 mg of purified, unmodified Band 3 per mL of resin (Boodhoo and Reithmeier, 1984). For initial studies of the interaction, detergent extracts of ghost proteins (rather than purified Band 3) were used. To prepare detergent extracts, 1 volume of ghosts was added to 5 volumes of 1% $C_{12}E_8$ in 5 mM sodium phosphate, pH 8, vortexed, and incubated at 4 °C for 30 minutes. The supernatant resulting after centrifugation at 100,000 x g for 30 minutes was used as the "$C_{12}E_8$ extract". Interaction with the pCMB-Sepharose in columns was performed at 0 to 4 °C as follows: 2 to 2.5 mL of the $C_{12}E_8$ extract of ghosts, containing approximately 1.5 mg total membrane protein, was applied to 1 mL of pCMB-Sepharose at a flow rate of 6 mL/hr. After application of the $C_{12}E_8$ extract, the resin was washed with 0.1% $C_{12}E_8$ in 50 mM sodium phosphate, pH 8, until the effluent had a negligible absorbance at 280 nm, and was then eluted with the rinse buffer containing either 0.1% 2-mercaptoethanol or 1 mM pCMBS. The protein contents of aliquots from each bulk fraction (the $C_{12}E_8$ extract, the unbound column flow through, and the 2-mercaptoethanol eluate) were analyzed by SDS polyacrylamide gel elecrophoresis.

The interaction of purified Band 3 with pCMB-Sepharose in-batch was performed as folows: 200 µL of a solution of aminoethyl-purified, NEM-blocked Band 3 (see Chapter II for a description of the purification) at a concentration of approximately 1 mg/mL in 50 mM sodium phosphate, pH 7.0, 0.1% $C_{12}E_8$ was added to 200 µL of washed, packed pCMB-Sepharose in a 1.5 mL microfuge tube. The mixture was mixed end-over-end for 5 minutes at room temperature, then centrifuged 30 sec in a microfuge. The supernatant was removed and the resin was washed 3 times with 1.2 mL 50 mM sodium phosphate, pH 7.0, 0.1% $C_{12}E_8$. Next, 200 µL of 0.1% 2-mercaptoethanol in 50 mM sodium phosphate, pH 7.0, 0.1% $C_{12}E_8$ was added, mixed for one minute, centrifuged to remove the resin, and the supernatant taken as eluate. Aliquots of the samples before addition to the resin, supernatant, and the eluate were taken for protein determination and gel electrophoresis. For calculations of protein recovery in the supernatant and eluate, correction for the two-fold dilution into the resin's included volume was made. Control experiments with Sepharose 4B were also performed, and resulted in no binding.

C. RESULTS

1. Quantitation of NEM Labeling After Treatment of Cells

We used Band 3 from cells that had been treated with NEM such that the sulfhydryls on Band 3 that are known to react with NEM under these conditions would be blocked. We first confirmed the quantitation of [^{3}H]NEM labeling of Band 3. Treatment of erythrocytes with [^{3}H]NEM as described resulted in incorporation of 98 nmol NEM per mg total membrane protein, comparable to (1.17x) the labeling obtained by Rao (1979). The fraction of the total radioactivity that was associated with Band 3 after SDS polyacrylamide gel electrophoresis (Laemmli, 1970) was 0.13, as determined from the radioactivity in solubilized gel slices. That is, 12.9 nmol NEM per mg membrane protein was in Band 3. Using 2.4 nmol Band 3/mg ghost protein (Lieberman and Reithmeier, 1983), the calculated stoichiometry is 5.37 mol NEM per mol Band 3. Purification of Band 3 from [^{3}H]NEM-labeled cells, using an affinity resin composed of a Band 3 inhibitor, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS), attached to an Affi gel 102 matrix (Pimplikar and Reithmeier, 1986), provided essentially pure Band 3 with 5.3 x 10^{-2} nmol NEM/mg protein. Using M_r=100,000 daltons, this gives 5.3 mol NEM/mol Band 3. These data confirm that our treatment of Band 3 with NEM results in quantitative labeling of all the NEM-reactive cysteine residues in Band 3 described by Rao (1979). The confirmation of the stoichiometry of NEM labeling is clearly important for our assumption, since the NEM-reactive residues also react with pCMBS (Rao, 1979). Since the NEM-reactive sulfhydryls are blocked, then our assumption is that the only sulfhydryl in Band 3 that is left available to react with sulfhydryl reagents, free, or on a matrix, is the NEM-unreactive sulfhydryl.

2. Measurement of Reaction of Sulfhydryl Reagents with Ghost Membrane Proteins and with Purified Band 3 in Detergent Solution

The reaction of Band 3 with two sulfhydryl reagents was measured in ghosts from cells that had been NEM-treated. We found 3.5 nmol DTNB-reactive sites per mg ghost protein. This would correspond to about 1 mol/mole Band 3, assuming that all the reactive sites are in Band 3. This is in agreement with the similar determination by Toon *et al.* (1985). The residue reacting with DTNB, however, is not necessarily in Band 3. Treatment of NEM-blocked ghosts with 0.5 mM [^{3}H]NEM (for 1 hr at 37 °C, in 5 mM sodium phosphate, pH 7.0) resulted in association of 3.2 nmol [^{3}H]NEM/mg membrane protein. Less than 0.1 mol/mol was associated with Band 3 in an SDS polyacrylamide gel. Thus, no NEM-reactive groups are available in Band 3 in ghosts prepared from NEM-treated cells.

Band 3 was purified from cells that had been treated with NEM (NEM-blocked Band 3). The reaction of purified, NEM-blocked Band 3, in a solution of 0.1% $C_{12}E_8$ in 50 mM sodium phosphate, pH 7.0, with three sulfhydryl reagents was studied. Reaction with a 20-fold molar excess of DTNB, at pH 7.0, was monitored by measuring the absorption of 2-nitro-5-thiobenzoate at 412 nm, and indicated 1.9 mol DTNB-reactive residues per mol Band 3. Because DTNB is generally accepted as being specific for sulfhydryl residues (Means and Feeney, 1971), this suggests there are 2 reactive cysteine residues per mole of Band 3 under these conditions. [^{14}C]pCMB treatment of purified, NEM-blocked Band 3, with 175 µM for 2 hr at room temperature, resulted in incorporation of 2.2 mol/mol Band 3.

Reaction of purified NEM-blocked Band 3 in detergent solution with [^{3}H]NEM resulted in incorporation of [^{3}H]NEM into Band 3. The dependence of the level of incorporation on the concentration of NEM (Figure VI.2) is similar for reaction under two conditions: for 1 hr at 37 °C, and for 2 hr at room temperature. Incorporation appears to

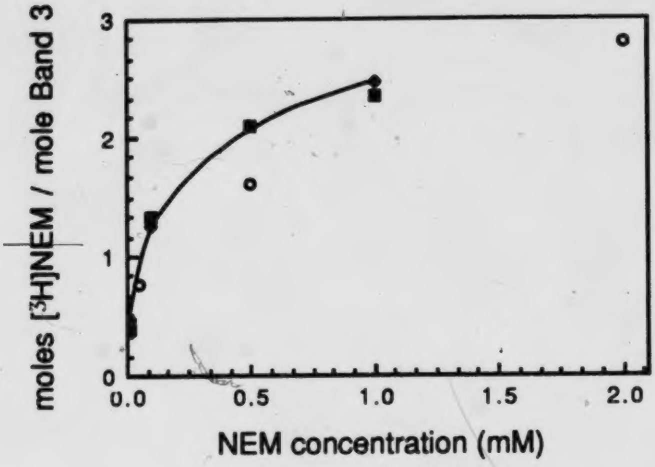

Figure VI.2 Concentration dependence of [^{3}H]NEM incorporation into purified, NEM-blocked Band 3. Band 3 was purified from NEM-treated cells. The purified protein in a 0.1% solution of $C_{12}E_8$, in 50 mM sodium phosphate, pH 7.0, was treated with [^{3}H]NEM at concentrations ranging from 0.01 mM to 2 mM, reacting either for 2 hr at room temperature (■) or for 1 hr at 37 °C (○). Treatment at room temperature was also carried out on the purified complex of NCh-60 and ChC-35 (◆). After removal of the unreacted reagent, the incorporation into Band 3 was measured as described in Methods.

saturate at higher concentrations of NEM, reaching a maximum of about 2.5 to 3 moles NEM per mole Band 3. After reaction with 0.5 mM [^{3}H]NEM and removal of the free NEM, there were no DTNB-reactive sites remaining, using the spectrophotometric determination. These results suggest that there are residues in Band 3 that are not reactive during NEM treatment of intact erythrocytes or ghosts, but which react with three sulfhydryl reagents, including NEM, in the detergent-solubilized protein.

The NEM label was distributed throughout the major proteolytic fragments of Band 3. Comparing the levels of incorporation obtained after treatment with 1 mM NEM for 2 hr at room temperature, intact Band 3 contained 2.4 mol NEM/mol Band 3, isolated TrC-55 contained 1.5 mol/mol, and isolated NCh-60 contained 1.5 mol/mol. This indicates stoichiometries of about 1 mol/mol each for NTr-41 and ChC-35, with about 0.5 mol/mol, by difference, in TrCh-17. Also, the radioactivity associated with the 17 kDa fragment after separation by SDS polycrylamide gel electrophoresis indicated about 0.5 to 0.7 mol/mol in this fragment (not shown), that is, it is clearly less than stoichiometric. The additional labeling outside the 17 kDa fragment must be due either to reaction with residues other than cysteine, or to incomplete reaction of the cysteines during the pre-treatment with unlabeled NEM.

3. Interaction of NEM-Blocked Band 3 with a Mercurial Affinity Resin

The Band 3 used to study the interaction of purified Band 3 in $C_{12}E_8$ with pCMB-Sepharose was about 90% pure, as can be seen in the gel lanes before application to the resin. The gel staining patterns of the fractions resulting from the interaction with pCMB-Sepharose are shown in Figure VI.3. The amount of protein that did not bind to the resin and remained in the supernatant was very low for both untreated and NEM-blocked Band 3: from 8 to 24 µg protein was measured in the supernatant for 200 to 224 µg protein applied to 200 µL resin. The amount contained in the eluate with 0.1% 2-mercaptoethanol was 144 to 152 µg, indicating a recovery of 67-75%. This data shows that Band 3 isolated from cells that have been treated with NEM can still bind to, and be eluted from, pCMB-Sepharose. Since free pCMB was shown to react with NEM-blocked Band 3, it is expected that it should react with the immobilized pCMB.

That the binding of Band 3 to the resin is mediated by covalent attachment of the protein to the immobilized pCMB is shown by the ability of the protein to be eluted from the washed resin by the addition of sulfhydryl reducing agents to the binding buffer. Several sulfhydryl reducing agents in addition to 2-mercaptoethanol were effective in eluting the Band 3, including 1 mM dithiothreitol, 10 mM cysteine, and 0.1% thioglycolic acid. The possibility that Band 3 is bound noncovalently to the pCMB is unlikely, since

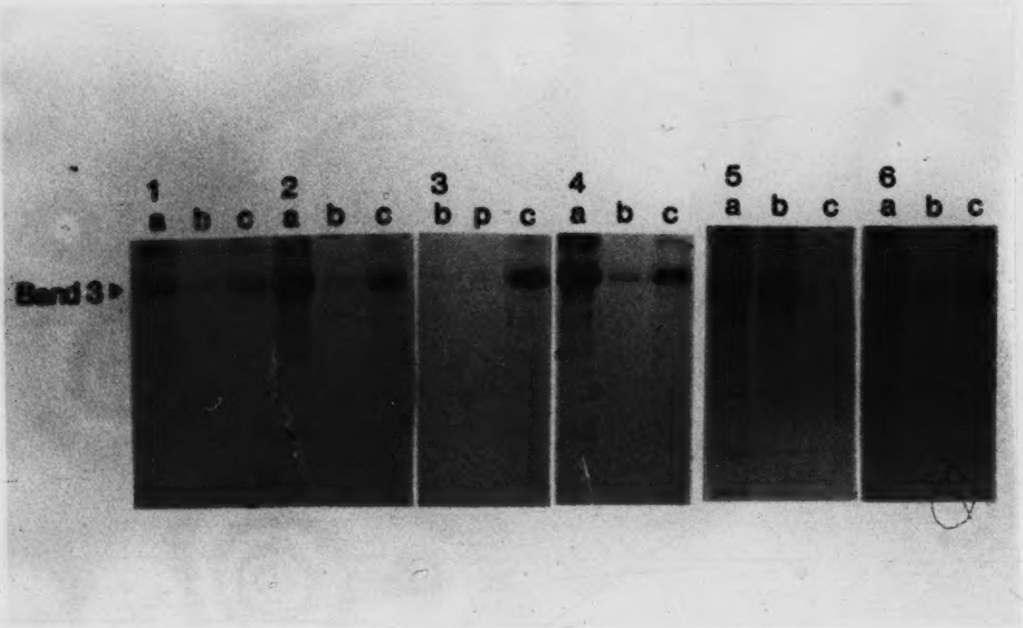

Figure VI.3. Interaction of purified Band 3 with pCMB-Sepharose after treatments with sulfhydryl reagents. Aliquots of equal volumes of the fractions resulting from interaction in-batch with pCMB-Sepharose were run on SDS polyacrylamide gels (10% acrylamide), and stained with Coomassie Brilliant Blue. The three fractions in all except group 3 are: **a**, Band 3 in 0.1% $C_{12}E_8$, 50 mM sodium phosphate, pH 7.0, before application to the resin; **b**, supernatant after incubation with the resin (the un-bound fraction); **c**, eluate with 0.1% 2-mercaptoethanol added to the rinse buffer of 50 mM sodium phosphate, pH 7.0, and 0.1% $C_{12}E_8$. Six different samples were tested for their interaction with the resin, each having been treated with a different combination of reagents, as such: **1**, untreated Band 3; **2**, NEM-blocked Band 3 (erythrocytes were twice treated with 12 mM NEM at pH 7.4, for 1 hr at 37 °C, prior to purification of Band 3); **3**, NEM-blocked Band 3 (as in set 2), for which the first fraction (**a**) is not shown, and where **p** is the eluate produced by 1 mM pCMB added to the rinse buffer, and **c** is 2-mercaptoethanol elution after the pCMB elution;. **4**, [^{14}C]pCMB-treated Band 3 (NEM-blocked Band 3, in $C_{12}E_8$ solution, treated with 175 µM [^{14}C]pCMB for 2 hr at 22 °C, and dialyzed before application to the resin); **5**, NEM-blocked Band 3 with 0.1% 2-mercaptoethanol present during the interaction with the resin; **6**, NEM-blocked Band 3, with about 12 mM pCMB present during interaction with the resin.

the structurally dissimilar 2-mercaptoethanol would not be expected to displace Band 3 by binding to a pCMB site in the protein. In addition, the non-sulfhydryl analog of 2-mercaptoethanol, ethyleneglycol, at a concentration of 1%, produced no elution of Band 3.

Free pCMBS up to a concentration of 10 mM was not able to elute the bound Band 3 from the pCMB-Sepharose. After application of NEM-blocked Band 3 to the resin, washing with 1 mM pCMB did not release any protein (Figure VI.3, 3 lane p). Subsequent treatment with 2-mercaptoethanol-containing buffer eluted Band 3, as before. 50 mM pCMBS eluted about 40 % of the amount of protein eluted by 2-mercaptoethanol (not shown). The ineffectiveness of pCMBS in eluting Band 3 could represent an enhanced stability of interaction with the immobilized ligand compared to the free ligand. Alternatively, perhaps once the NEM-unreactive residue has reacted with the immobilized pCMB its accessibility is limited, such that the larger organic mercurial pCMBS has more limited access than the other sulfhydryl reagents. A similar phenomenon has been described, in which a putative conformational change renders the pCMBS site less accessible to glutathione than to cysteine (Toon and Solomon, 1986).

To further characterize the interaction between Band 3 and the mercurial resin, the effect of treatment of Band 3 with free pCMBS prior to interaction with the resin, on the attachment of Band 3, was investigated. Ghost membranes containing NEM-blocked Band 3 were treated with pCMBS (1 mM, for 90 min, at 22 °C) and the ghosts were washed before detergent extraction. When this detergent extract was applied to pCMB-Sepharose, Band 3 appears to bind and be eluted as in untreated ghosts (not shown). Similarly, purified Band 3 that had been treated with 175 μM [^{14}C]pCMB to a stoichiometry of labeling of 2.2 mol/mol Band 3, as described above, was bound to and eluted from pCMB-Sepharose (Figure VI.3,4). In other words, attachment to the resin was not blocked by pre-treatment with pCMB or pCMBS. This observation is not fully unexpected, since the covalent mercaptide bond that a sulfhydryl forms with pCMBS is subject to competition by sulfhydryl reducing agents and to exchange with other mercurials (Means and Feeney, 1971).

Direct evidence for exchange was obtained by applying [^{14}C]pCMB-modified Band 3 from NEM-treated cells (in which free [^{14}C]pCMB had been removed by dialysis) to 1mL of pCMB-Sepharose in a column (Figure VI.4). The Band 3 protein bound to the column, with no protein detectable in the flow-through, while over half the radioactivity (60%) eluted with the flow-through. The evidence that the radiolabel found in the column flow-through was not simply noncovalently associated comes from the fact that when the same preparation was exposed to SITS-AffiGel, less than 20% of the radioactivity did not bind. Addition of buffer containing 0.1% 2-mercaptoethanol eluted Band 3 protein, as well as most of the remaining radioactivity, from the resin. The relative amounts of radioactivity and protein eluted corresponded to a stoichiometry of 0.96 mol [^{14}C]pCMB/mol Band 3. Our interpretation is that the pCMB on the resin would provide a ligand to compete with that attached to the residue on Band 3, and would displace a certain fraction of the pCMB (or pCMBS) on Band 3, thus binding the Band 3. Considering that the stoichiometry of [^{14}C]pCMB labeling was 2.2 mol/mol Band 3, the eluted Band 3 would be expected to have some label still associated if each Band 3 molecule interacted with the resin by a single reactive group.

Demonstration of competition for Band 3 binding to pCMB-Sepharose, by inclusion of competing sulfhydryl reducing agents, would further support that the interaction is due to mercaptide bond formation. When the Band 3 solution applied to the resin contained 0.1% 2-mercaptoethanol the interaction of Band 3 with the resin was fully prevented (Figure VI.3,5). 10 mM cysteine and 1 mM dithiothreitol were as effective as 0.1% 2-mercaptoethanol in blocking binding. When 1 mM pCMBS or about 12 mM pCMB was present in the Band 3 solution during its application to the pCMB-Sepharose

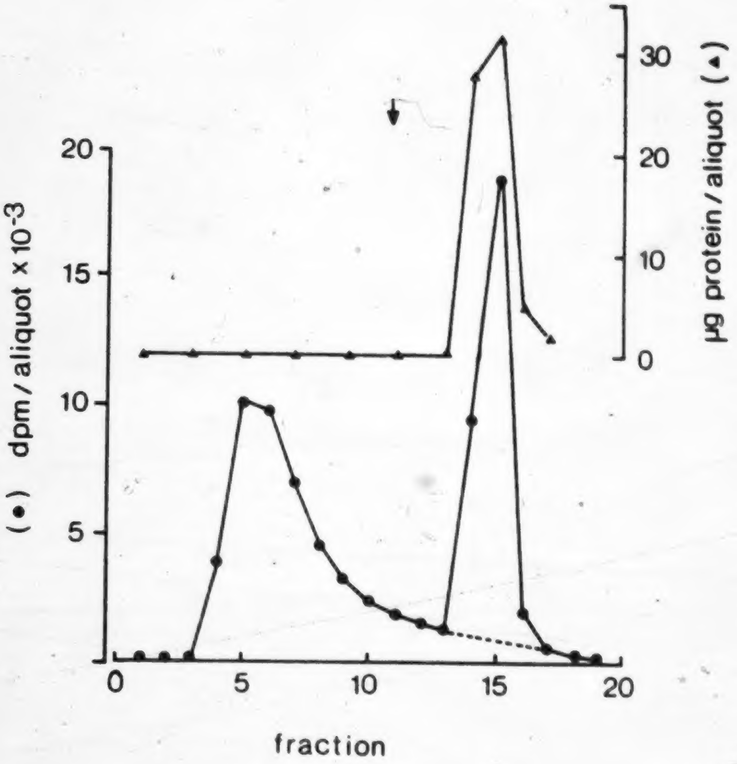

Figure VI.4 Displacement of [^{14}C]pCMB from Band 3 during chromatography on pCMB-Sepharose. Purified, NEM-blocked Band 3 was labeled with [^{14}C]pCMB to a stoichiometry of 2.2 mol/mol Band 3, and the free [^{14}C]pCMB removed by dialysis (as described in Methods). A portion of the [^{14}C]pCMB-modified Band 3 (0.4 mL at 1 mg protein/mL) was applied to a column containing 1 mL of pCMB-Sepharose, which had been washed at 4 °C with 50 mM sodium phosphate, pH 7.0, containing 0.1% $C_{12}E_8$. Application of the sample was followed by elution with 50 mM sodium phosphate, pH 7.0, containing 0.1% $C_{12}E_8$, then (as indicated by the arrow) with the same buffer containing 0.1% 2-mercaptoethanol. Aliquots of 50 µL of the fractions were taken for scintillation counting (●) and protein determination (▲). The extrapolated baseline (- - -) from the first peak was subtracted from the second before calculation of the stoichiometry.

the amount of Band 3 not bound to the resin, and appearing in the supernatant, was slightly increased, and elution from the resin was correspondingly somewhat diminished, relative to a sample without pCMB or pCMBS present (Figure VI.3, 6). The amount of protein not binding to the resin represented 17% of that recovered, compared to 8% unbound in only

NEM-blocked (set 2). A partial interference with binding, but never a complete prevention of binding, was observed in four such interactions. A concentration as high as 50 mM pCMBS gave qualitatively similar results (Lieberman, personal communication). A more complete prevention of binding was anticipated but it is possible that the immobilized ligand is in high enough concentration that substantial concentrations (up to 50 mM) of free ligand do not compete effectively to completely prevent binding. It is also important to consider that the expected affinity of the ligand may be altered by immobilization.

Band 3 from cells that had been treated with 100 μM DIDS (at 37 °C, for 30 min, at pH 7.4, producing complete reaction) in addition to the NEM treatment of the cells, also bound to and was eluted from the resin (data not shown). Labeling Band 3 with both NEM and DIDS therefore does not prevent the attachment of Band 3 to immobilized pCMB. This is consistent with the finding by Solomon of lack of effect of DIDS on pCMBS inhibition of water transport, and with a location of the pCMB-reactive residue outside the DIDS binding region (Solomon *et al.*, 1983).

D. DISCUSSION

This study was designed to address the reactivity and accessibility of the putative NEM-unreactive cysteine residue in Band 3. Both the measurements of binding to free sulfhydryl reagents and the interaction with immobilized pCMB that we have shown are direct measurements of covalent attachment of sulfhydryl reagents to Band 3, as distinct from noncovalent binding, or binding measured indirectly. The first important point is that this confirms that there is a residue in NEM-blocked Band 3 that reacts covalently with pCMB, and which is likely, but not unequivocally, a sulfhydryl. Our finding of reaction between Band 3 and immobilized pCMB at 4 °C, as well as at room temperature, agrees with the finding by Solomon *et al.* of [^{203}Hg]pCMBS labeling for treatment at 4 °C (Solomon *et al.*, 1983). Benga and coworkers, however, reported an absence of any labeling when reaction was at 0°C; some of their data (Benga *et al.*, 1986b) actually show about 2 nmol pCMBS per mg ghost protein for reaction at 0 °C for 15 min. It is possible that the low level of labeling that occurs at 4 °C may be significant in terms of the reactivity of a residue in Band 3, even though it is not correlated with the inhibition of water transport (Benga *et al.*, 1986a,b).

The interaction with matrix-bound pCMB also indicated that essentially all the Band 3 molecules contained the pCMB-reactive residues. However, the number of reactive residues per Band 3 molecule could not be determined from the studies of interaction with matrix-bound reagent. The measurement of reaction of free [^{14}C]pCMB with Band 3 indicated reaction of two moles pCMB per mole Band 3. The number of reactive residues remaining in NEM-blocked Band 3 was confirmed using two other sulfhydryl reagents, DTNB and NEM. It had been assumed that there was a single residue on Band 3 that remained available for reaction under these conditions, based on the total number of cysteines from previous amino acid analyses and the sequence data. The finding of reaction of free pCMB with two residues on NEM-blocked Band 3 makes interpretation of the results more complicated than if there were definitively a single reactive residue. The identity of the Band 3 residues reacting with free pCMB and with the pCMB-Sepharose is therefore ambiguous. We have demonstrated that NEM-blocked Band 3 binds covalently to immobilized pCMB, and that the interaction is likely mediated by either of two residues in the protein, but have not been able to demonstrate that it interacts *via* the single NEM-unreactive cysteine.

The results presented here indicate that there are two groups in Band 3 protein that, although unreactive toward NEM in cells, react with NEM, DTNB and pCMB in $C_{12}E_8$-solubilized Band 3. It is not likely, though, (and the protein chemical results do not support) that there are two cysteine residues that are unreactive to NEM in erythrocytes. Because the stoichiometry of cysteine residues in human Band 3 is five, our finding of stoichiometries of two sulfhydryl reagent sites in NEM-blocked Band 3 that become reactive upon solubilization must therefore involve at least one residue other than a cysteine residue. As mentioned earlier, pCMB and DTNB are generally thought to be specific for sulfhydryl residues (Means and Feeney, 1971). The simple interpretation that the three sulfhydryl reagents are all reacting with the same residues can not be correct. Although we have not determined the locations of the residues labeled with sulfhydryl reagents in our experiments, it is very likely that the NEM-"cryptic" cysteine is among the reactive residues.

The finding of NEM-reactive sites in NEM-blocked Band 3 that are exposed upon detergent solubilization is interesting. The idea that solubilization can expose previously unreactive residues is not new, though. A class of groups in erythrocyte membrane proteins that react with NEM only after solubilization with Triton X-100 was identified by Haest *et al.* (1981), but it was not determined in which proteins these groups were located. Our results show that some are in Band 3 protein, and suggest that the residues are inaccessible when the protein is in a membrane (either in cells or in ghost membranes). The possibility that the additional incorporation of [^{3}H]NEM after solubilization is due to reaction with the sulfhydryl in the 17 kDa fragment is attractive. Consideration of the results, though, suggests that perhaps this cysteine does not become reactive to NEM upon solubilization. The incorporation of NEM into TrCh-17 after treatment in detergent solution was estimated to be roughly 0.5 - 0.7 mol/mol at concentrations approaching saturation. The interaction with pCMB-Sepharose was not affected, however. This suggests that the pCMB-reactive residues (or at least some of them) are left intact after the second NEM treatment. If a cysteine is not made reactive upon solubilization, though, it is difficult to explain the observations that, after the additional NEM treatment of solubilized Band 3, we find no DTNB-reactive residues, and a partial reduction in the [^{14}C]pCMB labeling (not shown). It may be possible that pCMB (including immobilized) is reacting with other than sulfhydryl residues.

We suggest that the simplest interpretation of these results is that the cysteine corresponding to murine Cys-498 can mediate the interaction with pCMB-Sepharose, and that it does not become reactive with NEM upon solubilization of Band 3. To explain the labeling by NEM when Band 3 is in $C_{12}E_8$ we suggest that other types of residues, such as lysines, are reacting. The exposed sites are clearly not involved in the interaction with pCMB-Sepharose. Clarification of whether the cysteine residue in TrCh-17 has reacted with NEM is needed in order to interpret our results further. One possible approach to this information would be to determine the stoichiometries of the end products of acid hydrolysis of the NEM derivatives with cysteine and lysine. Both for the intact protein and the isolated TrCh-17 fragment, the differences in these products before and after the second treatment with NEM should indicate whether the cysteine residue in TrCh-17 has reacted. It would seem more direct to try to determine whether the reaction of pCMB is with cysteine, or with some other group. Localization of the pCMB-reactive residues even to labeled proteolytic fragments might be difficult, though, due to the lability of the mercaptide bond.

Whatever residue type is reactive with pCMB in detergent solution, our results provide information concerning the relative accessibility of the NEM-unreactive, pCMB-reactive residues. Our observation that NEM-blocked, purified Band 3 reacted with immobilized pCMB indicates that the NEM-unreactive residues with which pCMB reacts are accessible to within ≈ 12 Å (the distance from the matrix to the Hg) of the surface of $C_{12}E_8$-solubilized Band 3. Although this does not localize the residues relative to other residues, or even to a particular side of the membrane, it does indicate that they must not be buried very deeply inside the protein's structure in nondenaturing detergent solution. This observation is interesting, in view of the selective reactivity of the residues with sulfhydryl reagents.

Although our study does not directly address the role of NEM-unreactive, pCMB-reactive residue(s) in water transport inhibition, we can comment that our data is more consistent with the time course of reaction with pCMB as measured by fluorescence (Solomon *et al.*, 1983; Verkman *et al.*, 1986), than with the longer time courses observed for production of inhibition of water transport (Solomon *et al.*, 1983; Sha'afi and Feinstein, 1977; Macey, 1984). The continuing disagreement between the time courses of the phenomena occurring after pCMB(S) treatment suggests that the initial reaction of pCMB with Band 3 may not be the step that results in inhibiton of water permeability. The possibility has been suggested that the slow development of inhibition could be related to subunit rearrangement of Band 3 (i.e., dissociation of tetramers), based on evidence from: the sublimation of water from beneath intramembrane particles (IMPs) in freeze-fractured membranes (Pinto de Silva, 1973); the changes in both IMP distribution and water permeability noted in pathophysiological conditions (summarized by Benga, 1986b); and the single channel conductances measured after incorporation of Band 3 into planar bilayers, which were attributed to Band 3 tetramers (Benz *et al.*, 1984). In the same vein, it has been suggested (Haest *et al.*, 1981) that the NEM-unreactive cysteines might be located in the contact regions between oligomers. Until an unambiguous understanding of the reactivity of the NEM-unreactive cysteine in Band 3 is known, the interpretation of investigations addressing any functional roles of the cysteine residue will be vague.

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VII. GENERAL DISCUSSION AND PERSPECTIVES

The emphasis of the studies described in this thesis was dual, using two separate research approaches to investigate the structure and function of the anion exchange protein of human erythrocytes. The work has clarified the results of previous studies, and also opened up areas for future studies.

The aim of the first project, described in Chapters II and III, was to develop a procedure to reinsert purified Band 3 into vesicles in a preparation that would be suitable for both functional and conformational studies. The approach of carefully characterizing the purified protein in detergent solution before reconstitution, in order to assure retention of native protein structure, proved to be important. We noted alterations in the properties of binding of a transport inhibitor to Band 3 protein after exposure to high concentrations of octyl glucoside that suggested a functional alteration had occurred, along with some changes in the protein's secondary and quaternary structure. The change in inhibitor binding might help explain functional alterations noted in several of the previously reported reconstituted preparations. Our findings illustrate the importance of employing both structural measurements, and the most senstive measure of functionality as possible, in monitoring the protein prior to and during reconstitution. They also emphasize the general principle that the choice of detergent for both the purification and reconstitution steps is of utmost importance in obtaining a functionally reconstituted vesicle preparation, and must be made with care. This principle is generally accepted in theory, but not so widely applied in practice.

The circular dichroism studies presented in Chapter III are the first described for purified Band 3 in a membrane environment. Even with adequate elimination of or correction for spectral artifacts, the limitations of the methods of analysis of such spectra remain. Because the methods for analyzing far ultraviolvet CD spectra to determine the amounts of protein secondary structural elements use basis spectra of soluble proteins, they may not provide ideal estimates of the actual secondary structural content for a membrane protein. As is the known forté of circular dichroism measurements, such spectra can be most useful for detecting structural differences, e.g., as we have used the method in showing that there are not detectable alterations in the net secondary structure when the stilbenedisulfonate inhibitor, DIDS, is bound to Band 3. Further studies using reconstituted Band 3 are suggested, making use of differences in the CD spectra and extending measurements into the near UV range, to examine the effects of various substrates, and of membrane composition (i.e., cholesterol), on the conformation of Band 3. A collaborative effort between Dr. Reithmeier and Dr. Jung will continue.

The use of chemical modification to identify functionally important residues in anion exchange was limited to continuing an investigation of the transport inhibition by one carboxyl-selective reagent in particular, the carbodiimide ETC. The elucidation that there were two very distinct modes of inhibition of anion transport caused by this reagent (Werner and Reithmeier, 1988) led to a major re-evaluation of the previous work, and of our thoughts as to the mode of inhibition.

The mode of inhibition in which the reversal is stabilized by added nucleophiles, which is evidence for an important carboxyl residue in anion exchange, is interesting, since it points to a regulatory role of low pK_a amino acid residues outside the stilbenedisulfonate binding region. A role for residues that act to regulate or modify the translocation step of transport had been suggested previously only based on the pH dependence of the transport of several anions. This suggests the residues defined by this mode of inhibition are different from the glutamate residues characterized by Jennings (Jennings and Anderson, 1987), which were clearly in the stilbenedisulfonate site. The difficulty we experienced in finding conditions to obtain specific incorporation of a labeled nucleophile into Band 3 protein indicates further attempts at labeling may be difficult. The indications that the residues involved are distinct from those modified by Woodward's Reagent K makes it worthwhile to make further attempts to identify these residues. Perhaps the use of reconstituted Band 3 for the modification would be a useful approach, to avoid the interfering reactions with other proteins. The reproducibility of the transport characteristics in such vesicles would have to be ensured in order to adequately study the effects of modification on transport.

The discovery of ETC-activated citrate as a Band 3 protein-modifying reagent (Chapter IV) was fortuitous. The results on the characteristics of this modification are all consistent with, and suggestive of, the idea that the residue in Band 3 that is modified may be the DIDS-reactive lysine in the TrCh-17 fragment. Further experimentation to show this directly, by purification and sequencing of a small, labeled peptide, is indicated. The effort would be extremely worthwhile since the sequence of the human protein around the DIDS-reactive residues is not known. Approaches to this problem are suggested in Chapter IV. If it turns out that modification is at one of the potential DIDS-reactive lysines, this modification would add to the understanding of the functional role of this residue in transport. This information may be of limited use, since the modification may perturb the environment around the modified lysine. If modification is not of these lysines, then it represents a new site of modification that inhibits transport.

Finally, the study described in Chapter VI uses chemical modification and sulfhydryl affinity chromatography to investigate the reactivity and accessibility of sulfhydryl groups in Band 3. Our evidence suggests that residues in Band 3 may become reactive to NEM upon solubilization of Band 3 with the nondenaturing detergent $C_{12}E_8$. Modification of these sites, however, does not affect the covalent attachment of Band 3 to an immobilized pCMB matrix. We conclude that a sulfhydryl group in Band 3 mediates the attachment of the protein to immobilized pCMB and that this sulfhydryl is likely to be the NEM-unreactive cysteine present in the 17 kDa TrCh-17 membrane fragment. Because NEM penetrates the bilayer, a constraint on the reactivity of Band 3 to NEM beyond membrane-protection needs to be considered. The suggestions that the reactivity may be related to the oligomeric state of Band 3 raises very interesting questions about the oligomeric interactions, and about a possible role of Band 3 oligomers in osmotic water permeability. The idea of oligomers of Band 3 possibly mediating the water transport is an intriguing possible solution to the mystery of how to accomodate the masses of work indicating the water transport inhibition site possibly being located on Band 3, while the water permeation occurs by a separate pathway from anion transport. Testing this suggestion will likely require experimental approaches beyond those used here, but may be aided by, for example, using gel filtration to study the effect of modification with pCMB on the oligomeric state of Band 3 in detergent solution.

The potential use of the immobilized sulfhydryl reagent to study the very important question of on which side of the membrane the NEM-unreactive cysteine is exposed has been limited by the preliminary findings of nonspecific reaction of Band 3 with pCMB, as well as technical problems relating to interaction of proteins and vesicles with the resin. Determining the sidedness of this residue would be of great use in limiting models of the folding of Band 3 in the membrane. If specific labeling conditions could be developed, the use of the matrix-bound mercurial, in conjunction with Band 3 in vesicles, might be a feasible approach.

In some respects, several of the studies described were an exercise in the disadvantages of using the classical approach of chemical modification: most reagents are not specific for a single type of amino acid side chain, and there are numerous possible side reactions. The disadvantages are further complicated for membrane proteins: when treatments are done in membranes many other proteins can be modified; when done in detergent solution, the structure of the protein may not be native, and residues that would not normally be accessible in the native state may be exposed. These both point to the possible advantages of attempting future chemical modification studies of the anion transporter on reconstituted Band 3. This might be done in conjunction with spectroscopic measurements of the protein's structure.

Now, with the existence of a cloned cDNA for Band 3, at least for the murine form, it is exciting to anticipate that application of the techniques of site-directed mutagenesis will complement the approach of chemical modification in studying functionally important residues. This should not mean that chemical modification will be left behind -- the value and efficiency of using protein chemical results to select candidates for sites for mutagenesis is clear. Although continued efforts at getting useful information about Band 3 structure from CD measurements have been suggested, it is realized that the information coming from such studies will not be complete. Such measurements are most useful when interpreted in conjunction with a model of the 3-dimensional structure of the protein. A concerted effort at determining the crystal structure of Band 3 is a noble research goal, and one which may be fruitful, considering the advances in crystallization of other integral membrane proteins.

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